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Synthetic Peroxidases

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INTRODUCTION

For a number of years it has been generally accepted that the enzyme peroxidase is composed of a specific protein united to protohematin (1, 2, 3, 4). However, conclusive evidence has been furnished by Theorell (4). He separated horse-radish peroxidase into peroxidase I and peroxidase II by cataphoresis. At pH 7.5 peroxidase I migrated to the cathode, while peroxidase II went to the anode. Peroxidase I was precipitated by picric acid and had absorption bands at 583 and 548 $m\mu$. Peroxidase II was not precipitated by picric acid and had absorption bands at 640 and 498 $m\mu$. Theorell was able to split peroxidase II into a protein and hemin by treatment with acetone-HCl at -15°C . After neutralization the peroxidase could be put together again by adding a solution of protohematin to the protein. The activity came back slowly and the absorption bands did simultaneously. This reversible splitting did not succeed with peroxidase I.

Theorell's separation of peroxidase into hemin and protein and the re-synthesis of peroxidase has been fully confirmed in this laboratory, although the starting material consisted of a mixture of peroxidases I and II, since we did not possess the apparatus needed for the separation of these two enzymes through electrophoresis. It was thought of interest to employ apoperoxidase and to make use of Theorell's demonstration of its ability to unite with hematin in order to see whether metalloporphyrins composed of metals other than iron, i.e., Cu, Co, Mn and Ni, could unite to form substances with peroxidase activity. We thought it of interest also to find out if modified iron porphyrins when united with the apoenzyme had any peroxidase activity.

EXPERIMENTAL PART

Preparation of Peroxidase

Fresh horse-radish root was washed well, cut into slices and pounded to a pulp in a hammer mill. The pulp was neutralized to give a pH of 6-7. Next the juice in the pulp was pressed through canvas in a Buchner press at 250 atmospheres. The extract was then centrifuged and filtered. For every 100 cc. of filtrate, 60-65 g. of solid ammonium sulfate were added. This precipitated all of the peroxidase, which then was filtered off by gravity at icebox temperature. The precipitated peroxidase was dialyzed for 48 hours in an icebox. The dialyzed material was neutralized to pH 7 and then filtered. For every 100 cc. of filtrate 28-30 g. of solid ammonium sulfate were added, after which the preparation was filtered. The precipitate was discarded and for every 100 cc. of filtrate 35 g. of solid ammonium sulfate were added. The precipitated peroxidase was filtered off and was dialyzed against distilled water until all salt had been removed. To the dialyzed solution an equal volume of ice-cold 95 per cent ethyl alcohol was added. This mixture was placed in the icebox for twenty-four hours, and then centrifuged. The precipitate was discarded. To the supernatant liquid was added the same volume of 95 per cent ethyl alcohol as before. This precipitated all the peroxidase, which came down as a gelatinous brown precipitate. It was centrifuged off, and the supernatant discarded. The precipitate was dissolved in as little water as possible and dialyzed in the cold for twenty-four hours against distilled water containing a few cc. of 0.1 *M* acetate buffer of pH 5-5.5. The dialyzed material was filtered and the filtrate used for the following experiments.

Preparation of Apoenzyme

Five cc. of the peroxidase were cooled to 2-3°. To this solution were then added, while mixing rapidly, 100 cc. ice-cold acetone containing 1 cc. of concentrated HCl. The mixture was placed in the icebox for ten minutes during which time it was mixed well. It was centrifuged and the precipitate was washed with a little ice-cold acidified acetone. It was centrifuged again and the white precipitate was dissolved in 20 cc. of 0.013 *M* Na₂HPO₄. This solution had no peroxidase activity, was very slightly colored, showed no absorption bands with the spectroscope and when treated with dithionite and pyridine gave no indication of pyridine hemochromogen bands. The acetone washings gave definite bands for acid hematin and a strong test was obtained for pyridine hemochromogen.

Activity Determination

The most frequently used method for the determination of peroxidase activity is that of Willstatter and Stoll (5). This method takes considerable time. Consequently a comparative method was developed which was rapid and easy to carry out. This was as follows: 10 cc. of a saturated solution of guaiacol in H_2O were mixed with 1 drop of peroxidase solution delivered from a standard pipette into a colorimeter cup. To this solution was added 0.20 cc. of hydrogen peroxide (0.6 per cent) and the solution was mixed immediately. A stop-watch was started. The cup was placed in a colorimeter and the time required for the darkening test to match a standard iodine solution (0.029*N*) was determined. This method was not used for absolute values but only as a comparative method. With not too great dilutions there is a straight line relationship between peroxidase concentrations and time in seconds to match the standard. In each experiment the most active preparation (usually hematin + apoenzyme) was called 100 per cent, while the others were given their comparative values. Example: Preparation A took 12 sec. to match the standard. Preparation B took 22 seconds to match the same standard. Consequently the activity of B as compared with A was: $12/22 \times 100 = 54.5$ per cent.

Preparation of Metalloprotoporphyrins

The first series of experiments was conducted to determine what effect a substitution of cobalt, nickel, manganese or copper for the iron in hemin would have on the peroxidase activity when these new compounds were combined with the apoenzyme.

Hemin was prepared from defibrinated cow's blood, according to Fischer (6), and recrystallized (7), from chloroform and quinine. The protoporphyrin was prepared from hemin according to Fischer and Pützer (8), with a few modifications. The protoporphyrin was recrystallized from chloroform and pyridine. The metals (Co, Ni, Mn, Cu) were introduced into the protoporphyrin in glacial acetic acid (8). All of these metalloprotoporphyrins were obtained in the crystalline state except the manganese protoporphyrin. No reference to the previous preparation of this compound has been found.

Apoenzyme Combinations of the Metalloprotoporphyrins of Mn, Co, Cu, and Fe

The metalloprotoporphyrins were dissolved in 0.1 *M* NH_4OH-NH_4Cl buffer. This was found necessary since the metalloprotoporphyrins of

nickel and copper are quite insoluble in any other suitable solvent. For each of the metalloprotoporphyrins 3 different concentrations were made up in 10 cc. of NH_4Cl buffer. Different fractions of 1 cc. from the above solutions were added to 1 cc. of the apoenzyme so as to give

TABLE I

	Metalloprotoporphyrin added	Time to match standard av of three determinations	Activity as compared with most active preparation
	<i>mg</i>	<i>sec.</i>	<i>per cent</i>
Ferriprotoporphyrin chloride (hemin) plus 1 cc. apoenzyme	0.4	37.8	37.8
	0.2	17.6	81.0
	0.1	14.3	100.0
	0.02	62.4	22.9
	0.01	113.1	12.6
	0.002	272.0	5.3
Cobalt protoporphyrin plus 1 cc. apoenzyme	0.4	960.0	1.5
	0.2	760.0	1.9
	0.1	920.0	1.6
	0.02	960.0	1.5
	0.01	1,020.0	1.4
	0.002	1,000.0	1.4
Manganeseprotoporphyrin plus 1 cc. apoenzyme	0.4	600.0	2.4
	0.2	616.9	2.3
	0.1	576.0	2.5
	0.02	50.6	28.4
	0.01	272.0	5.3
	0.002	608.0	2.3
Copperprotoporphyrin plus 1 cc. apoenzyme	0.4	800.0	1.8
	0.2	672.0	1.9
	0.1	808.0	1.8
	0.02	740.0	1.9
	0.01	790.0	1.8
	0.002	820.0	1.7

different concentrations of the metalloprotoporphyrin. The difference in volume of the solutions was made up by adding pure 0.1 M NH_4OH - NH_4Cl buffer. Since we found the apoenzyme to be unstable upon standing, it was necessary to prepare new apoenzyme for every experiment. After the metalloprotoporphyrins had been added to the apo-

enzyme, the mixture was placed in the icebox for twenty-four hours. Then activity determinations were run. The combinations of cobalt and copper protoporphyrins with the apoenzyme had no peroxidase activity (Table I). On the contrary, the manganese compound showed some activity.

Further experiments were run, comparing nickel and cobalt protoporphyrin compounds of apoenzyme with ferriprotoporphyrin apoenzyme. These had no activity (Table II).

A curious phenomenon was observed both with hematin + apoenzyme and manganese protoporphyrin + apoenzyme which showed up con-

TABLE II

	Metalloprotoporphyrin added	Time to match standard av of three determinations	Activity as compared with most active preparation
	<i>mg.</i>	<i>sec.</i>	<i>per cent</i>
Ferriprotoporphyrin chloride (hemin) plus 1 cc. apoenzyme	0.2	44.4	36.8
	0.02	16.4	100.0
	0.002	59.2	27.8
Nickelprotoporphyrin plus 1 cc. apoenzyme	0.2	608	2.7
	0.02	824	2.0
	0.002	728	2.2
Cobaltprotoporphyrin plus 1 cc. apoenzyme	0.2	672	2.4
	0.02	728	2.2
	0.002	736	2.2

sistently. There is an optimum concentration of the metalloprotoporphyrin as regards activity, above and below which the activity decreases. This is contrary to what one would expect. However, the enzyme preparation is no doubt impure, which might give rise to discrepancies.

We next investigated the peroxidase activity of the ferrimesoporphyrin compound with apoperoxidase.

Preparation of Ferrimesoporphyrin Chloride

Ferrimesoporphyrin chloride was made according to Harrison (9), using palladinized charcoal as catalyst in saturating the vinyl groups in hemin with hydrogen.

Apoenzyme plus Ferrimesoporphyrin Chloride and Ferriprotoporphyrin Chloride

Ferrimesoporphyrin chloride had low solubility and only dissolved in the NH_4Cl buffer after long standing. Two different concentrations were made in 10 cc. of the buffer for each of the two compounds. Different fractions of 1 cc. were added to 1 cc. of the apoenzyme and the difference in volume was made up by adding pure NH_4Cl buffer.

TABLE III

	Metalloprotoporphyrin added	Time to match standard av. of three determinations	Activity as compared with most active preparation
	<i>mg.</i>	<i>sec.</i>	<i>per cent</i>
Ferrimesoporphyrin chloride plus 1 cc. apoenzyme	0.300	105.5	9.3
	0.120	16.9	58.0
	0.080	9.8	100.0
	0.040	10.0	98.0
	0.012	16.8	58.5
	0.008	27.7	35.2
	0.004	64.0	15.3
Ferriprotoporphyrin chloride plus 1 cc. apoenzyme	0.300	37.6	26.0
	0.120	16.3	60.0
	0.080	20.1	48.7
	0.040	38.5	25.5
	0.012	138.8	7.0
	0.008	173.5	5.6
	0.004	265.0	3.7

The results given in Table III indicate that the apoenzyme + ferrimesoporphyrin chloride forms a compound which has a greater activity than hematin + apoenzyme. The same phenomenon mentioned previously was also found here, namely, that there is optimum concentration of prosthetic group below and above which the activity decreases. Over the whole range, the ferrimesoporphyrin chloride + apoenzyme had a higher activity than hematin + apoenzyme. The results were confirmed in another experiment.

It appeared of interest to test out the compound of ferrihematoporphyrin with apoperoxidase.

Preparation of Ferrihematoporphyrin Chloride

Ferrihematoporphyrin chloride was prepared according to Küster and Bauer (10), by making the dimethyl ether of hematoporphyrin and then introducing the ferric chloride. The product was semi-crystalline and its solubilities and spectroscopic picture checked with those of previous workers. However, this product, when added to apoenzyme, showed no peroxidase activity. Next, we employed some hematoporphyrin ferric chloride which was prepared by introducing ferric chloride into hematoporphyrin (8) instead of into the dimethyl ether. This compound dissolved more readily in the NH_4Cl buffer than did the compound prepared

TABLE IV

	Metalloporphyrin added	Time to match standard av. of three determinations	Activity as compared with most active preparation
	<i>mg.</i>	<i>sec.</i>	<i>per cent</i>
Ferrihematoporphyrin chloride plus 1 cc. apoenzyme	0.05	15.6	75.0
	0.04	15.3	76.0
	0.02	21.0	56.0
	0.01	36.5	32.0
Ferriprotoporphyrin chloride (hemin) plus 1 cc. apoenzyme	0.05	13.2	89.0
	0.04	11.7	100.0
	0.02	12.1	97.0
	0.01	13.2	89.0

from the hematoporphyrin dimethyl ether. As Table IV shows, this ferrihematoporphyrin chloride when united with apoenzyme possesses peroxidase activity, but it is somewhat less active than the peroxidase made from ferriprotoporphyrin chloride (hemin) and apoenzyme.

SUMMARY

1. The work of Theorell (4), in which he was able to split peroxidase II reversibly into a protein part and a hematin part has been confirmed. Further experiments have been carried out in which porphyrins containing copper, cobalt, manganese and nickel have been employed instead of hematin (iron porphyrin). Of these metalloprotoporphyrins, the only one showing any ability to function as the prosthetic group of peroxidase has been that of manganese. The synthetic manganese protoporphyrin

compound was found to possess 20–30 per cent of the activity of re-synthesized peroxidase.

2. A curious phenomenon which has been noticed is that there is an activity optimum for the percentage of added prosthetic group.

3. Preliminary results indicate that a synthetic peroxidase formed by allowing ferrimesoporphyrin chloride to unite with apoenzyme is even more active than the peroxidase formed from ferriprotoporphyrin chloride + apoenzyme. The preparation formed by adding ferrihematoporphyrin chloride to apoenzyme was somewhat less active than the ferriprotoporphyrin chloride + apoenzyme.

We wish to express our gratitude to both the Sage Fund and to the Rockefeller Foundation for grants supporting this research.

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A Method for Measurement of Yeast Growth in Bios and Vitamin Investigations

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It has been established that bios consists of at least five separate factors. The five (inositol, pantothenic acid, biotin, thiamin, and pyridoxin) are also members of the group of B vitamins which are required by higher animals. Inositol, pantothenic acid and biotin were known as yeast growth substances before their importance in animal nutrition was recognized. The isolation and identification of these vitamins was materially aided by analytical methods based on yeast growth procedures.

There is reason to believe that the specific biochemical reactions in which the vitamins participate may be the same in both the higher and lower forms of life. For this reason it may be expected that yeast experimentation will also contribute to the eventual elucidation of vitamin physiology. A convenient method of measuring the extent and rate of yeast growth should, therefore, be of interest at the present. Furthermore, the vitamin content of foods and tissues can be determined by yeast growth methods and the technique described in the present communication is believed to be especially suitable for these assays. In this method yeast is grown in standard 18 mm. pyrex test tubes and the concentration of yeast suspended in the medium is directly estimated with a photoelectric colorimeter without the necessity of opening the tubes to remove samples. In this way any number of readings can be made during the growth period of a single test. Reading is rapid, 15 seconds per tube or less, and therefore a great number of tests can be made simultaneously. During the growth period the tubes are shaken in an air thermostat by means of a shaking apparatus designed for use in the Kahn test. Both the photoelectric colorimeter and the shaker are relatively inexpensive and are obtainable as standard items from various supply houses.

Thorne and Bishop (1) studied various methods of estimating yeast concentration in suspensions and recommended the turbidometric or nephelometric methods for rapidity and accuracy. The nephelometric method requires sampling and dilution of the yeast suspension and is therefore not suitable for rapid and continuous observations on many tests. Thorne and Bishop built their own photoelectric turbidimeter and with this apparatus Thorne (2) was able to follow yeast growth directly, without taking samples, using, as culture vessels, special flat sided bottles of 160 ml. capacity. The present method accomplishes the same object with standard equipment. Williams, MacAlister and Roehm (3) earlier described a turbidometric method which employed a specially constructed thermocouple but few other investigators have undertaken to build this type of apparatus.

APPARATUS

The Colorimeter. The Lumetron 400¹ fitted for use with 18 mm. O.D. test tubes is employed with white light suitably reduced in intensity by a filter made of a gray glass and wire screen combination. The glass transmits 20% of incident light and the screen 33%. This combination is supplied as part of the regular complement of filters if requested. White light was chosen as the light source because a series of tests showed that it was absorbed least by the colored solutions ordinarily encountered in plant and animal extracts. The percentage of white light absorbed by simple yeast suspensions was found to be as high as with any filter except the blue filter but blue light was particularly highly absorbed by the yellow to brown plant extracts tested.

The Test Tubes. A dozen matched test tubes are supplied with the instrument but many more than twelve are needed. From a stock of pyrex test tubes (18 × 150 mm. with lip) all those were selected which had an outside diameter of 17.8 to 18.0 mm. inclusive when measured one inch from the bottom, with the diameter corresponding to the path of the light beam. The tubes were then cleaned and filled with distilled water. The light transmission of each was then compared with any one tube chosen at random and set at 90% transmission. Tubes which showed more than 1% deviation in transmission were rejected. As a further check the tubes were compared when filled with a dilute molasses solution of about 50% transmission. About half of the tubes in stock were found to be satisfactory in dimension and transmission.

¹ Manufacturer—Photovolt Corp., N. Y. C.

For greater convenience in handling the tubes the lips were cut off and the ends fire-polished. Culture tubes of the same size (without lips) were tried but found to be unsatisfactory.

The Shaker. During the growth periods the test tubes are shaken in a shaking machine devised for the Kahn test.² Since Kahn test tubes are much smaller than 18 mm. in diameter the regular tube holders were not obtained but a piece of soft pine $19 \times 11\frac{1}{8} \times 2\frac{3}{8}$ inches was drilled with holes $\frac{7}{8}$ inch in diameter and 2 inches deep. The holes were purposely made larger than the test tubes to permit vigorous shaking which prevents the yeast from settling out. Properly spaced one hundred holes can be made in the wood block.

Temperature Control. Growth tests are conducted at 30°C. by placing the entire shaking apparatus in an incubator or air thermostat.

EXPERIMENTAL

Calibration Curve. The relationship between yeast concentration and per cent absorption may be used for the estimation of yeast concentration. In all measurements the test tubes contain 10 ml. of suspension, although as little as 7 ml. can be used if necessary. A sample calibration curve is shown in Fig. 1. The light intensity is adjusted to 100% transmission by means of a rheostat and with a tube containing pure solvent in the light path. This tube remains in the machine and can be moved into the light path as frequently as desired to check the constancy of the light source. Light absorption is an approximately linear function at low yeast concentrations. At intermediate absorptions (30 to 80%) a straight line is obtained if the log of yeast concentration is plotted against per cent light absorption. This property of the curve may prove useful in vitamin assay procedures.

Yeast Growth. Yeast growth experiments may be roughly divided into two types depending on whether the extent of growth is measured or the rate of growth is observed. Fig. 2 illustrates the results obtained in a series of tests wherein the extent of growth was progressively limited by the quantity of ammonium nitrogen present in the medium. Hourly observations were made until growth ceased as indicated by no change in the light absorption, i.e., 20 hours at 30°C. If the quantity of yeast grown is estimated by reference to the calibration curve, Fig. 1, a direct relationship is observed to exist between milligrams of ammonium sulfate and milligrams of yeast (see Fig. 3).

² The Fisher-Kahn shaker—Eimer and Amend—Fisher Scientific Company, Manufacturers.

Some substances affect the rate of growth and these can be studied in much the same fashion. Fig. 4 gives the results of measurements

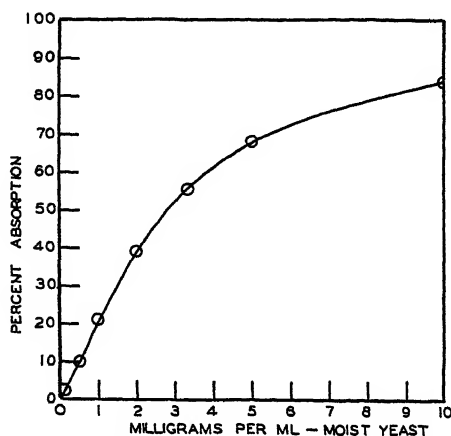


FIG. 1. Relationship between yeast concentration and light absorption.

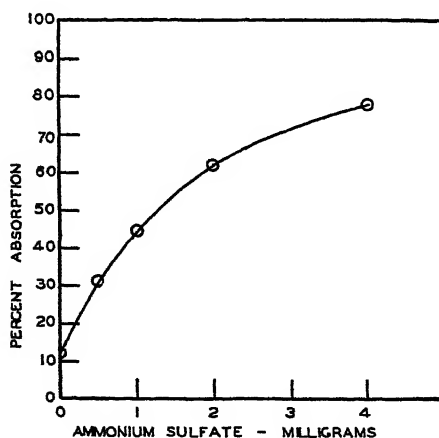


FIG. 2. Relationship between ammonium sulfate and light absorption.

made every 2 hours on a pair of cultures both of which contained all of the known bios factors except that a quantity of hydrolyzed vitamin-free casein was added to one (Curve 2).

Bios Requirements of Yeast. Yeast strains may differ in their growth factor requirements. These differences may be detected and studied

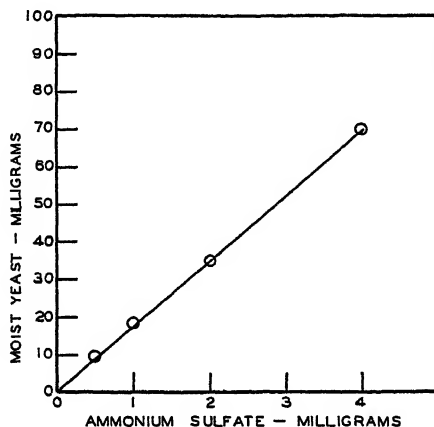


FIG. 3. The extent of yeast growth as determined by the quantity of ammonium sulfate.

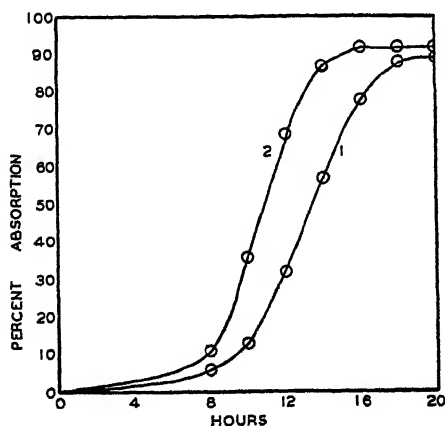


FIG. 4. Curve 1. "Complete" basal medium. Curve 2. "Complete" medium plus hydrolyzed casein.

by the present technique. Figs. 5, 6, and 7 show the behavior of three different yeast types. In all cases the medium was basically a "syn-

thetic" one which contained dextrose, vitamin-free hydrolyzed casein, inorganic salts, potassium citrate buffer, inositol, calcium pantothenate

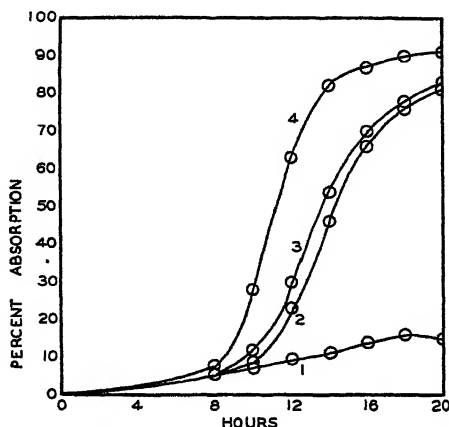


FIG. 5. Growth of a type A yeast. Curve 1. Basal medium plus inositol, calcium pantothenate and biotin. Curve 2. Same as 1 plus thiamin. Curve 3. Same as 1 plus pyridoxin. Curve 4. Same as 1 plus thiamin and pyridoxin.

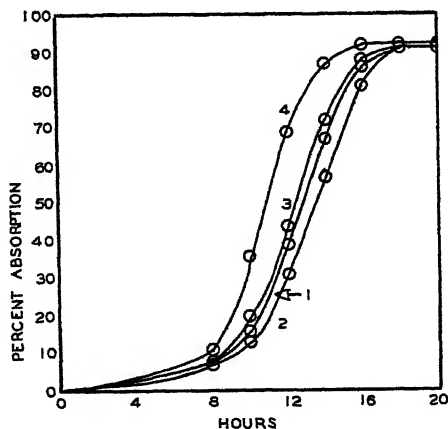


FIG. 6. Growth of a type B yeast. Composition of media the same as in Fig. 5.

and crystalline biotin. The seeding rate was 0.1 mg. of moist yeast per 10 ml. As may be observed, type B yeast was the only yeast which

gave a good growth on the basal medium. Type A yeast showed a greatly improved growth when either thiamin or pyridoxin was added to the medium, whereas, type C yeast required only vitamin B₆ and was not aided by thiamin. In every case the best growth was obtained when all five vitamins were present in the medium. These three types of yeast were originally defined by Schultz, Atkin, and Frey (4) at a time when only impure concentrates of biotin were available. Further studies of these important differences between yeast strains are under way.

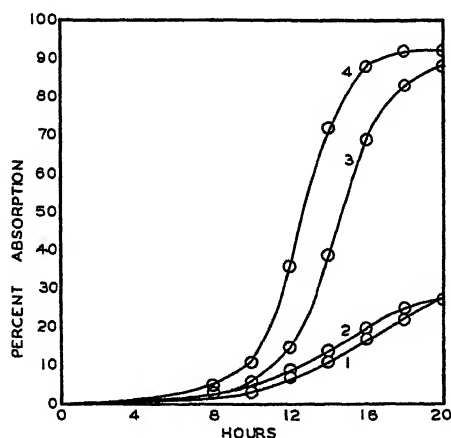


FIG. 7. Growth of a type C yeast. Composition of media the same as in Fig. 5.

SUMMARY

1. A method is described whereby the extent or rate of yeast growth may be estimated in suspensions without the necessity of removing samples.

2. The method employs a photoelectric colorimeter and a shaking machine, both being manufactured items and relatively inexpensive.

3. Growth tests are made in 18 mm. pyrex test tubes with a medium volume of 10 ml.

4. The measurement of yeast concentration, the extent of yeast growth and the rate of yeast growth, and the observation of the specific bios requirements of yeast strains are illustrated by typical experiments.

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The Carotenoid Pigments of the Fruit of *Celastrus scandens* L.

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Technology, Pasadena**

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The intensely red berries of *Celastrus scandens* ("False Bitter-Sweet") contain a complicated polyene pigment mixture (chiefly esters) which can be resolved by the Tswett chromatographic method. By this procedure we have isolated the carotenoids listed below which contain different types of chromophores, viz., that of β -carotene (No. 1-3), γ -carotene (No. 4), torulene (No. 6-7) and a new type (No. 5).

1. β -Carotene, $C_{40}H_{56}$, (about 3% of the total pigment).
2. A crystalline ester which on hydrolysis yielded cryptoxanthin, $C_{40}H_{55}\cdot OH$ (1%).
3. An ester of zeaxanthin, $C_{40}H_{54}(OH)_2$, characterized by the unusual, thread like form of the crystals. It is clearly different from the well known dipalmitate, physalene (1), and is very probably zeaxanthin-di-n-caprylate (main pigment of the fruit, 80%).
4. A crystalline ester from which a monohydroxy- γ -carotene, perhaps rubixanthin (2), $C_{40}H_{55}\cdot OH$, was obtained (1%).
5. A polyene of unknown structure but showing the longest wavelength spectral maxima hitherto reported, as far as the authors can determine for any carotenoid: 587, 537.5 and 503 $m\mu$, in carbon disulfide (0.2%).
6. A hydrocarbon possessing the same spectrum as the pigment No. 7 (0.5%).
7. A remarkable component of the berries (15%) is a new polyene alcohol termed celaxanthin, $C_{40}H_{55}\cdot OH$ (or $C_{40}H_{53}\cdot OH$). Its natural,

* Contribution No. 888.

crystalline ester yielded the free alcohol in well crystallized form. Both are characterized by relatively high wave length spectral maxima, indicating 12-13 conjugated double bonds. In carbon disulfide the maxima are located at $16\text{ m}\mu$ longer wave lengths than those of lycopene. On the other hand the spectrum of celaxanthin, in all solvents investigated, is identical with that of torulene which was isolated by Lederer (3) from a red yeast, *Torula rubra*. The two compounds possess very probably identical chromophors while the adsorption affinities are considerably different because of the presence of a free hydroxyl group in celaxanthin. Both pigments show the phenomenon of spontaneous or catalyzed isomerization (4).

The structure of celaxanthin cannot be given with certainty but the following considerations suggest a possible formula.

(a) An ozonization experiment showed the formation of 0.55 moles of acetone per mole pigment, indicating the presence of one isopropylidene end group.

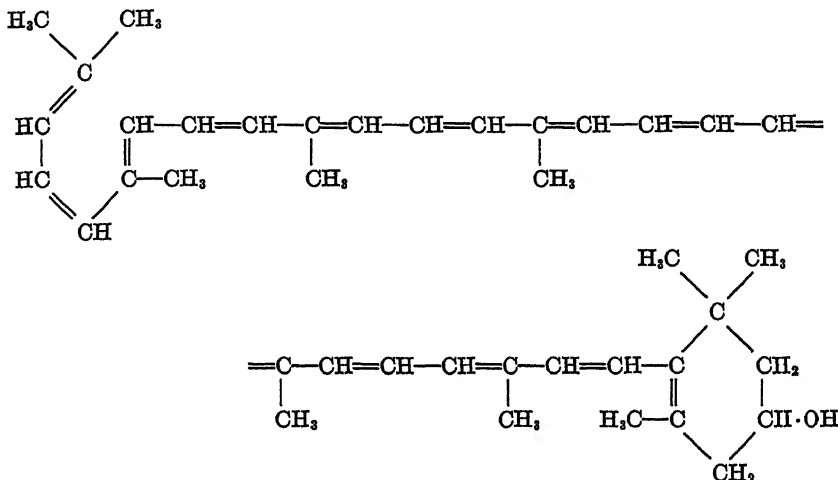
(b) The longest wave length maximum in carbon disulfide solution is for lycopene $548\text{ m}\mu$ and for γ -carotene $533\text{ m}\mu$, indicating that in similar cases cyclization shifts this maximum by about $15\text{ m}\mu$ toward the violet. If the difference between rhodoviolascin, $\text{C}_{40}\text{H}_{54}(\text{OCH}_3)_2$, (Karrer and Solmsen 5) and celaxanthin is not in the number of conjugated double bonds but in the fact that the former pigment has two acyclic, the latter only one acyclic end group, then the expected longest wave length maximum for celaxanthin is $573 - 15 = 558\text{ m}\mu$. The observed value is $562\text{ m}\mu$. On the other hand if each aliphatic conjugated double bond increases the wave length of the maximum mentioned by about $20\text{ m}\mu$ (cf. crocetin and bixin), the addition of two such double bonds to the acyclic end of the γ -carotene chromophor should give the figure $533 + 40 = 573\text{ m}\mu$ which is $11\text{ m}\mu$ higher than the observed value.

(c) Celaxanthin does not show enolization and therefore structures with $=\text{C}(\text{OH})-$ are excluded.

On the basis of the foregoing considerations and analytical data a tentative celaxanthin formula is given, corresponding to a dehydro-rubixanthin. Less probable is a formula with a symmetrical distribution of the 13 conjugated double bonds throughout the molecule as suggested by Karrer and Koenig (6) for rhodoviolascin, which would involve an unusual position for the hydroxyl group in celaxanthin.

Lederer's torulene may be a methoxy-celaxanthin-methylether. A

close relationship between the bacterial pigment rhodoviolascain, the yeast pigment torulene, and celaxanthin, occurring in a high plant, is evident on spectroscopic basis.



Celaxanthin(?).

Acknowledgement. The authors are indebted to Professor C. B. Van Niel for a pure culture of *Torula rubra*, to Professor J. Bonner for bacteriological advice and to Dr. G. Oppenheimer as well as to Mr. G. Swinehart for microanalytical assistance. The isopropylidene estimation was kindly carried out by Mr. W. A. Schroeder.

EXPERIMENTAL

Calcium hydroxide (Shell Brand Chemical hydrate, 98% through 325 mesh) was used as an adsorbent in practically all chromatographic operations. Elutions were carried out with a petroleum ether-alcohol mixture 3:1 if pure petroleum ether (b.p. 60–70°) or the same solvent containing some acetone had been used as a developer. After developing with benzene or benzene and acetone, a benzene-methanol mixture 3:1 is the most suitable eluent. "Saponification" in the following text signifies that the pigment solution has been kept in contact with concd. methanolic potassium hydroxide over night, then washed alkali-free and dried with sodium sulfate. The pigment concentrations were determined with a Pulfrich Gradation Photometer (Zeiss, light filter S47) and the spectra with an Evaluating Grating Spectroscope (Zeiss; light

filter BG7). Spectral data refer to petroleum ether solutions unless otherwise indicated.

The starting material was obtained in South Dakota. 790 g. of the air-dried fruit gave 300 g. of stems and hulls, 260 g. of seeds and 230 g. of intensely red flesh. Only the latter was worked up. The material was twice extracted by shaking mechanically for 15 minutes with a two-phase mixture of 800 cc. of petroleum ether and 150 cc. of methanol. After drying at 40° it was possible to grind the residue in a coffee mill. It was extracted again in the same manner. The pigment of the combined extracts was transferred into petroleum ether by addition of water, and washed alcohol-free in an automatic apparatus described recently by one of the authors (7). The solution was then dried with sodium sulfate and concentrated in vacuo as far as possible. A solution of the residue in 500 cc. of petroleum ether was developed on lime in a percolator (40 × 15 × 8.5 cm.) with 1 liter of petroleum ether, then with 2 liters of the same solvent containing 10% acetone, and finally with 0.7 liter containing 20% acetone. More than a dozen zones appeared which can best be considered in five sections:

Section I, in addition to the strongly adsorbed top layers, included the polyene mentioned above with the extraordinarily long wave length spectrum, (in carbon disulfide: 587, 547.5 (503) m μ , in petroleum ether: 542.5, 506, (474) m μ , and in alcohol: 541, 503 m μ). After saponification it showed the behavior of a dihydroxy-compound in the partition test and did not give any color reaction with strong hydrochloric acid.

Section II consisted of esterified celaxanthin and some of its stereoisomers. Section III contained some esters of zeaxanthin and of a hydroxy- γ -carotene, closely followed by a hydrocarbon showing the celaxanthin spectrum. Section IV was practically homogeneous and consisted of the main pigment (zeaxanthin ester). Section V contained only a small fraction of the total pigment, chiefly cryptoxanthin-ester and β -carotene; 7.5 mg. and 17 mg. of crystals respectively, were isolated. The cryptoxanthin was identified by hydrolysis, crystallization, spectra and mixed chromatogram. Below the β -carotene zone and well separated from it, a much lighter epiphasic pigment appeared which has been obtained so far only as an orange oil showing strong yellow fluorescence in ultraviolet light. The maxima were at 462, 433 m μ and after the addition of iodine at 460.5, 431.5 m μ .

Only the investigation of Sections II, III, and IV will be described in detail.

Investigation of Section II

This section of the column was composed of an intensely red upper zone and a lighter pigment below it. Both were eluted separately and developed with benzene on smaller columns. The lighter pigment separated into two components (minor layers were discarded). The three main pigments obtained in this manner were eluted and precipitated from benzene with methanol. As no definite crystal forms could be observed, all the solid material (85 mg.) was combined again and saponified. After washing and evaporation, the pigment was dissolved in a little benzene. The solution was diluted with petroleum ether and developed on calcium hydroxide with benzene containing 10% acetone. The main zone contained only celaxanthin and was followed by a heterogeneous zone of its stereoisomers.

Celaxanthin. The main zone yielded 9.2 mg. of celaxanthin on elution and crystallization from carbon disulfide (or benzene) and ethyl alcohol. This carotenoid forms a dark red crystal powder, macroscopically not unlike lycopene (crystallized from petroleum ether and alcohol). Under the microscope long needles are visible, partially grouped in rosettes or bundles. M.p. 209–210° (cor., in a Berl block, sealed tube, filled with carbon dioxide). Another sample melted at 204–205°. For analysis the compound was dried in high vacuum at 45° for 40 minutes. We were unable to obtain completely ash-free preparations even after repeated recrystallizations and washing of the benzene solution with doubly distilled water. In the results of the following analyses corrections have been made for 1.0% ash.

<i>Analysis.</i>	$C_{40}H_{56}O$.	Calculated	C 86.69,	H 10.22.
	$(C_{40}H_{54}O)$.	"	" 87.21,	" 9.88.)
		Found	" 86.90, 87.31,	" 9.83, 10.46.

14.0 mg. of celaxanthin gave on ozonization and hydrolysis an amount of acetone corresponding to 1.57 cc. of *N*/20 iodine, and after subtraction of the blank value (0.23 cc.): 1.34 cc. Found: 0.55 isopropylidene groups per mole.

Celaxanthin is moderately soluble in benzene or carbon disulfide at room temperature, considerably less soluble in petroleum ether, and practically insoluble in methyl or ethyl alcohol. When partitioned between light petroleum and 85% methanol, it is epiphasic but with 95% methanol an appreciable fraction appears in the lower phase. Due to the color intensity we were unable to determine whether the compound was optically active. Spectral maxima: in carbon disulfide: 562, 521, 487,

455 $m\mu$, in alcohol: 520.5, 488, 455 $m\mu$, and in petroleum ether: 520, 486.5, 456, (429) $m\mu$, (after the addition of iodine: 518, 485, 454, (429) $m\mu$).

Celaxanthin does not show noticeable isomerization when kept at 5° for 15 hours while solutions of its stereoisomers contain some celaxanthin after such treatment. If a benzene solution of celaxanthin is kept at 70° for 30 min. and developed on lime with benzene containing 20% acetone, the chromatogram shows three stereoisomers without colorless interzones. The process is reversible. The isomers are termed neocelaxanthin A, B, and C. Their spectra are given in Table I. The isomers A and C are formed in small quantities and are markedly less stable than neocelaxanthin B. The approximate composition of the equilibrium mixture was established by submitting either celaxanthin itself or its most stable isomer (B) to heating or to iodine catalysis. The

TABLE I

Spectral Maxima of Celaxanthin and Some of Its Stereoisomers and of the Equilibrium Mixtures Obtained by Iodine Catalysis in Benzene ($m\mu$)

Pigment	Before the addition of iodine				After the addition of iodine (equilibrium mixture)		
Celaxanthin.....	538.5	500	466	(436)	535.5	497.5	463
Neocelaxanthin A	534	497	464	(433.5)	535	497.5	464
Neocelaxanthin B	530	493.5	460	(431.5)	535	497.5	463.5
Neocelaxanthin C	536	496.5	461	(432)	535	496.5	460.5

final solutions were then chromatographed in each case and the colorimetric ratio of the zones formed was established (Table II).¹

For the comparison of the naturally occurring celaxanthin ester with torulene the latter was prepared in the following way. *Torula rubra* was grown on a 2% agar medium, containing 1% malt, 1% dextrose and suspended calcium carbonate, at 25°, in dim light for several weeks and then exposed to intense diffuse light for two days near a window. This increases the pigment intensity as observed by Lederer. The red surface was scraped off, dehydrated with methanol and extracted five times with mechanical shaking using methanol and petroleum ether in two phases. After every second extraction the mass was ground in a mortar. The

¹ Beside the isomers listed, 2-3% of the starting material appeared on the column as an irreversible top layer with no definite spectrum. Furthermore, about $\frac{1}{2}$ of the initial color intensity disappeared during the operations.

pigment was transferred into petroleum ether and saponified. Upon removing the alkali by washing, the pale solution deposited an amorphous pink precipitate which was centrifuged. The solution was concentrated, diluted with 1 vol. of benzene and developed on calcium hydroxide with the same solvent. The main zone (torulene) was followed by a small zone (neo-torulene) both of which were eluted separately. Spectrum of torulene in carbon disulfide: 565.5, 524.4, 490.5, (459) $m\mu$, in benzene: 540, 502, (469) $m\mu$, and in petroleum ether: 520.5, 487, 457.5 $m\mu$ (after the addition of iodine: 518.5, 485, 454.5 $m\mu$). A direct comparison with celaxanthin ester revealed no spectral differences. Neotorulene in benzene has the maxima: 535, 497, 464 $m\mu$ (with iodine: 537, 498, 464 $m\mu$). When developed with benzene on calcium hydroxide torulene is

TABLE II

Relative Photometric Values of Celaxanthin and Its Stereoisomers as Formed by Isomerization of Celaxanthin or Neocelaxanthin B

The figures denote percentage if the total color intensity of the isomerized mixture is = 100%

Starting material	Treatment	Relative photometric values			
		Celaxanthin	Neo A	Neo B	Neo C
Celaxanthin.....	Heat	59	7	26	8
Celaxanthin.....	Iodine	56	10	25	9
Neocelaxanthin B		52	11	24	13

adsorbed well below celaxanthin ester and still further below free celaxanthin.

Investigation of Section III

This section was divided into two parts; the upper one when re-chromatographed on lime from benzene, separated into two main zones (and many minor lines which were neglected) while the lower portion of Section III showed one important zone only. Each of the three main pigments was eluted, washed, dried, and evaporated. The three residues were treated as follows (in the sequence of decreasing adsorbability).

(a) A crystallization of the residue from hot petroleum ether gave an ester and this in turn yielded 22 mg. of zeaxanthin on saponification.

(b) Crystallization of the residue from petroleum ether and methanol gave 9 mg. of an ester which on saponification gave 3 mg. of long red

needles grouped in sheaves. M.p. 140° (cor.). The behavior in the partition test was like that of lycoxanthin or cryptoxanthin and an analysis showed the presence of one oxygen atom. The spectra were identical with those of γ -carotene, indicating the structure of a monohydroxy- γ -carotene: In carbon disulfide: 533.5, 496.5, 462.5, 434.5 $m\mu$, in petroleum ether: 496, 463, 434.5 $m\mu$ (after the addition of iodine: 492, 460, 431 $m\mu$). The compound is adsorbed on calcium carbonate from petroleum ether; it does not separate from lutein on calcium hydroxide when developed with benzene but does so on calcium carbonate. On lime it is located between zeaxanthin and lycopene.

(c) The evaporation residue gave 2.6 mg. of a crystalline but not completely pure pigment, the behavior of which indicated the composition of a hydrocarbon. In benzene: (535.5), 504, 474 $m\mu$ (on addition of iodine the spectrum became much sharper: 537, 499.5, 467, (437.5) $m\mu$).

Investigation of Section IV

The pigment was transferred from the eluate into a small volume of petroleum ether and crystallized by the addition of several volumes of methanol. The yield was 457 mg., m.p. $111-112^{\circ}$ (cor.), i.e. considerably higher than that of physalien. A mixed melting point with physalien showed that the two were not identical. The compound has a fibrous structure which is apparent macroscopically. The microscope shows a hair-like arrangement of winding threads. Recrystallization does not alter this form. The ester is readily soluble in petroleum ether but insoluble in methanol. On partition it is completely in the upper phase even if 95% methanol is used. It is most probably zeaxanthin-di-n-caprylate $C_{40}H_{84}(OCO \cdot C_7H_{15})_2$. For the purpose of analysis the crystals were dried in high vacuum, at 50° for 20 min. They were free of ash

<i>Analysis.</i>	$C_{86}H_{84}O_4$.	Calculated	C 81.90,	H 10.31.
		Found	" 82.38, 82.47,	" 10.25, 10.21.
<i>Mol. wt.</i>		Calculated	821.	Found 819, 781 (in camphor).

In accordance with the latter figures a colorimetric determination showed that the compound is 1.43 times weaker in color intensity than zeaxanthin. This corresponds to a molecular weight 815. The spectral maxima were identical with those of zeaxanthin, e.g. in petroleum ether: 486, 454 $m\mu$. The adsorption affinity was equal to that of physalien from which the compound did not separate on lime when developed with benzene. The position on this adsorbent is between γ -carotene and cryptoxanthin.

On saponification zeaxanthin, m.p. 207–208° (cor.), was formed and identified.

Analysis. $C_{46}H_{56}O_2$. Calculated C 84.44, H 9.93.

Found " 84.88, " 9.70.

Mol. wt. Calculated 568. Found 501 (in camphor).

The ethereal, saponified solution of 105 mg. of the crystalline ester described above was repeatedly extracted with water. The acid component was then transferred from the water layer into ether by means of sulfuric acid, traces of which were removed from the ether by washing. The pale yellow oily residue, remaining after evaporation of the ether smelled like caprylic acid. After several recrystallizations (at first with the addition of charcoal) the anilide showed a constant melting point 57° (cor.). It gave a depression with the anilides of n-capric and n-pelargonic acid.

SUMMARY

In the pigment of the berries of *Celastrus scandens* L. a complicated carotenoid mixture occurs, about $\frac{2}{3}$ of which is a new zeaxanthin ester. Most of the other pigments have spectral maxima at longer wave lengths than those of lycopene. Celaxanthin, $C_{40}H_{56}\cdot OH$ (or $C_{40}H_{56}\cdot OH$), a new polyene which constitutes $\frac{1}{3}$ of the total pigment, has been isolated and described. This compound as well as the red yeast pigment torulene shows the phenomenon of reversible isomerization. The spectra of torulene and celaxanthin are identical, on which basis a tentative structural formula for celaxanthin is given. A minor pigment of *Celastrus* exhibits the highest wave length spectrum hitherto reported for any carotenoid and has the first maximum at 586 m μ in carbon disulfide.

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Heat Capacity and Bound Water in Starch Suspensions*

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In a series of calorimetric measurements made on gelatin gels by Hampton and Mennie (3) the sum of the heat capacities calculated for the dry gelatin, ice, water, and sample container was greater than the observed heat capacity of the whole system. The authors at first attributed this discrepancy to the water portion of the sample; and suggested that, while the free water had a normal specific heat, the bound water might have a specific heat less than 1.00. Their data indicated a specific heat of 0.80 for bound water over temperature ranges well below zero to room temperature. Kotukov (6) similarly assigned specific heats ranging from 0.678 to 0.771 to the bound water in gum arabic sols. It was suggested that such a value for the specific heat of bound water might afford a new method for its estimation in lyophylic systems.

Subsequently, however, Hampton and Mennie (4) and Horn and Mennie (5) found that, in the temperature range 0° to 25°C., the sum of the calculated heat capacities was less than the observed total heat capacity. The calculation of specific heat for bound water from these data led to improbable values of 1.00 to 1.30. Consequently, these authors concluded* that other factors contribute to the divergence of the theoretical and observed heat capacities. In their discussion of these results they particularly indicate the possibility that heat effects accompanying the adsorption and desorption of water might be primarily responsible. They suggested that when the temperature of the system is raised from 0° to 25°C., some of the bound water is desorbed. This desorption of water is accompanied by the absorption of heat amounting to 6.7 cal. per gram of dry gelatin.

Apparently this effect is by no means limited to gelatin gels and gum arabic sols since similar results have been obtained in this laboratory with mixtures of raw starch and water and with dextrin sols. On the

* Contribution No. 445 of the Massachusetts Agricultural Experiment Station.

other hand, the heat capacities of mixtures of coarse sand and water, in which the adsorption of water should be small, did not show any appreciable divergence from the calculated values. It seems probable that this heat effect may be generally expected in lyophilic systems and further experiments are in progress to ascertain this point. Some additional features appeared in the starch-water data that seemed noteworthy. A linear relation was found between the observed and calculated heat capacities, for certain portions of the curves relating composition to heat capacity. A close relationship was noted between the bound water content and the observed heat capacities.

EXPERIMENTAL

Determination of Heat Capacity. Potato starch was prepared by grinding peeled raw potatoes to a pulp, from which the starch was washed with water, and then repeatedly washed with distilled water. The starch-water samples containing less than 30 per cent starch were prepared by weighing calculated quantities of starch and water directly into watertight copper tubes. The samples containing more than 40 per cent starch were taken at intervals from a mass of slowly drying starch. 500 g. of starch were suspended in distilled water and poured into a large shallow glass tray. After the starch had settled, the excess water was poured off and the mass of starch was slowly dried at room temperature with frequent and thorough mixing. Samples for heat capacity and for moisture content were taken simultaneously. Frequent checks indicated satisfactory uniformity of the starch-water mixtures. At still higher concentrations of starch, the drying was carried out in an oven at 37°C. and finally in a vacuum oven. The sample containers were copper tubes, holding 30 ml., securely closed with screw caps and composition washers. Tests revealed no leakage between 0°C. and 40°C. if the caps were tightened carefully. The samples were held in a rapidly stirred ice bath for several hours and then quickly transferred to quart vacuum bottles, containing 500 g. of distilled water. The vacuum bottles, fitted with stirring rods and Beckmann thermometers had previously been brought to equilibrium near 40°C. in an air thermostat. The vacuum bottles containing the samples were immediately returned to the thermostat and stirred until the contents came to equilibrium. The temperature of the thermostat was manually adjusted to keep pace with the downward course of the temperatures within the vacuum bottle.

Corrections for the gain of heat during transfer of the samples, for the heat capacities of the stirring device, thermometers, etc., and for the temperature lag of the vacuum bottles were accumulated in a correction factor determined by carefully standardizing the procedure with sample tubes filled with distilled water. Each value recorded in Table 2 is the average of at least four successive determinations on the same sample.

Determination of Bound Water. Bound water in a few samples was determined by the cryoscopic method of Newton and Gortner (7). The results, recorded in Table 1, gave an average value close to 0.30 grams of bound water per gram of dry starch.

The dilatometer method of Jones and Gortner (2) gave somewhat higher re-

sults because of the difficulty in removing all of the dissolved gases from the water suspension of the sample. Upon freezing the sample this gas appeared as bubbles in the ice with a corresponding error in the volume. Since repeated freezing and thawing, even under reduced pressure, failed to eliminate all the bubbles in the ice, it was assumed that these results for bound water would be high. No exhaustive search was made for the cause or the remedy of this error but one experiment indicated a possible explanation. Clear ice containing no air bubbles was placed in the dilatometer and covered with toluene. The pressure was reduced with a water pump and the sample of clear ice was thawed and then frozen. Upon freezing, air bubbles appeared in the ice layer. The only apparent source of this gas was from, or through, the toluene layer. Possibly appreciable amounts of gas remained dissolved in the toluene even at low temperatures and reduced pressure. When the ice was melted, a portion of this gas diffused from the toluene layer into the water layer where it appeared as bubbles upon re-freezing.

TABLE 1
Average Values for Bound Water in Starch-Water Suspensions

	Dumanski method	Cryoscopic method	Dilatometer method
Grams of bound water per gram starch.	0.31 \pm 0.02*	0.34 \pm 0.03	0.36 \pm 0.05
Per cent bound water	23.0	25.4	26.5

* This is an average of about 50 determinations with several samples of potato starch; run at different concentrations of sucrose, starch; and run at several temperatures.

Dumanski's (1) refractometer method proved to be admirably suited to starch-water suspensions. The technique described by Dumanski using an Abbe refractometer, however, allowed experimental errors in the sucrose determinations that were too large for a satisfactory calculation of the bound water. Consequently, a Zeiss immersion refractometer was used with calibration curves and temperature correction curves constructed from measurements on carefully prepared sucrose solutions. With these refinements sucrose concentrations were determined within ± 0.01 per cent with satisfactory agreement in the bound water calculations. In Table 1 are summarized the bound water contents of starch-water suspension by these methods.

EXPERIMENTAL RESULTS

The heat capacities of a series of samples ranging from 20 per cent starch to carefully dried starch (vacuum oven) were calculated from the experimental data by the following equation:

$$W(T_2 - T_3) \cdot F = C \cdot h_{cu} \cdot (T_3 - T_1) + R \cdot h \cdot (T_3 - T_1) \quad (1)$$

or

$$h = \frac{W(T_2 - T_3) \cdot F - C \cdot h_{cu} \cdot (T_3 - T_1)}{R \cdot (T_3 - T_1)} \quad (1a)$$

where W = grams of water in the calorimeter
 F = calorimeter factor
 C = weight of the copper sample container
 R = weight of starch-water suspension
 h_{cu} = heat capacity of copper
 h = heat capacity of sample
 T_1, T_2, T_3 = temperatures of ice bath; original, and final temperatures within the calorimeter, respectively.

The observed values recorded in Table 2 are the average of at least four determinations on each sample. The relation between the observed heat capacities and the starch content of the samples is shown by the points in Fig. 1. From these data, an equation can be obtained that describes the linear portion of the curve relating the heat capacity to the composition of the samples. Equation 2 expresses this relationship for starch samples from 0 to 77 per cent starch and the heat capacities calculated from this equation are recorded in the last column of Table 2.

$$H_r = h_s \cdot f_s + h_w \cdot f_w + 0.0875 \cdot f_s \quad (2)$$

H_r = heat capacity of the sample

h_s, h_w = heat capacities of dry starch and water

f_s, f_w = fractions of starch and water in the sample

No attempt was made to derive an equation for the heat capacities of the samples from 77 to 100 per cent starch because of uncertainties regarding the probable form of this part of the curve. Since the heat capacities in this region seem to be determined in part by an adsorption reaction, the curve may or may not be an exponential function. Therefore it was felt that the data were insufficient to clearly establish the form of this portion of the curve.

However, the theoretical relationship between heat capacity of the sample and its composition is expressed by the following equation 3 according to Robinson (8).

$$H_r = h_s \cdot f_s + h_w \cdot f_w \quad (3)$$

The values for H_r calculated from this formula are recorded in the third column of Table 2 and represented by the solid line in Fig. 1.

In Fig. 1 the break in the curve that includes the observed points appears at 77 per cent starch. This is the point where all the water in the sample is bound according to the data of Table 1. The coincidence of these points has suggested an intimate connection between the abnormal heat capacities of the samples and the water binding proper-

ties of the starch. Accordingly an explanation of the divergence between observed and theoretical values was sought in the water relationships of the sample.

TABLE 2
Observed and Calculated Heat Capacities of Starch-Water Suspensions

Starch content	Observed heat capacity	Theoretical heat capacity*	Calc. heat capacity**
<i>per cent</i>	<i>cal. per degree</i>	<i>cal. per degree</i>	<i>cal. per degree</i>
18.0	0.915	0.877	0.893
25.9	0.850	0.823	0.845
34.9	0.807	0.761	0.791
50.5	0.691	0.654	0.698
58.6	0.647	0.599	0.650
65.0	0.605	0.555	0.612
65.3	0.608	0.553	0.610
69.4	0.580	0.525	0.585
72.7	0.562	0.502	0.565
73.3	0.569	0.498	0.544
75.9	0.550	0.480	0.547
77.5	0.538	0.469	0.537
78.6	0.505	0.462	
79.9	0.498	0.453	
81.0	0.483	0.445	
83.4	0.464	0.420	
84.5	0.454	0.421	
86.8	0.423	0.405	
90.2	0.409	0.382	
91.1	0.385	0.375	
96.8	0.330	0.337	
97.3	0.328	0.333	
100	0.315		
100	0.311		
100	0.315		

* Calculated from equation 3.

** Calculated from equation 2.

As Hampton and Mennie (4) have pointed out, the assignment of heat capacities greater than 1 to bound water would be difficult to defend, although such an assumption would correct the discrepancy between the observed and theoretical values. The more plausible explanation, advanced by these authors, that the abnormal heat capacities are due to the adsorption or desorption of water as the temperature is changed,

is strongly supported by a slightly different treatment of the data in Table 2. The composition of the sample, calculated in grams of water associated with 1 g. of starch, was compared with the heat capacity of the water associated with 1 gram of starch. These heat capacities were calculated as the total heat capacity for a 40° rise in temperature

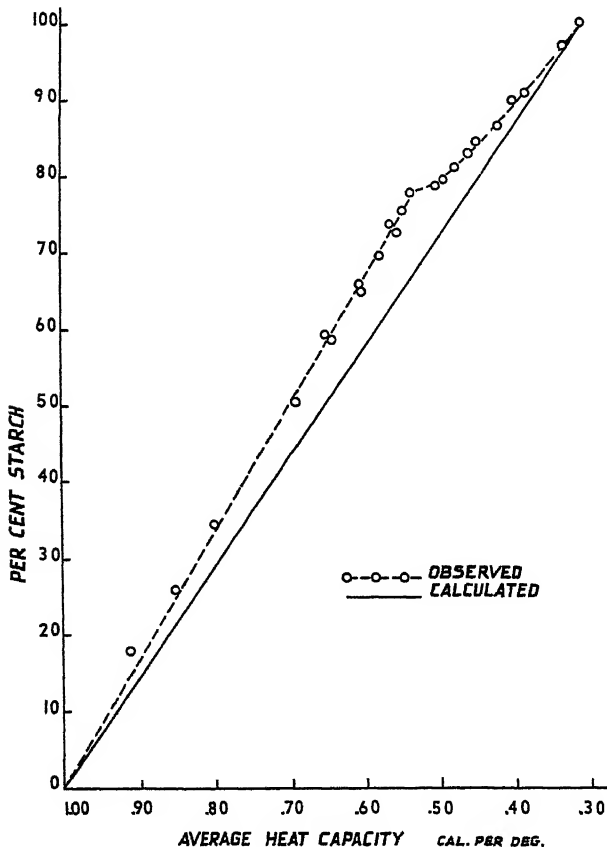


FIG. 1. Observed and calculated heat capacities for starch-water suspensions

since the point in question deals with the desorption of bound water over this temperature range. This data has been recorded in Table 3 and in Fig. 2. The grams of water associated with 1 g. of starch were obtained directly from the composition of the samples. The observed heat capacity of the water associated with 1 g. of starch was obtained

by dividing the observed heat capacity of each sample (Table 2, column 2) by the fraction of starch in that sample. From this heat capacity of the material that contains 1 g. of dry starch was subtracted the heat capacity of 1 g. of dry starch. This result multiplied by 40 gives the observed heat capacity of the water associated with 1 g. of starch as the

TABLE 3

Observed and Calculated Heat Capacities (0° to 40°C.) of the Water Associated with 1 Gram of Starch

Starch content	Water per gram starch	Observed heat cap. of water assoc. with 1 g. starch	Theoretical heat cap. of water assoc. with 1 g. starch*
<i>per cent</i>	<i>grams</i>	<i>cal.</i>	<i>cal.</i>
18.0	4.53	190.7	182.3
25.9	2.86	118.7	114.5
34.9	1.87	79.9	74.6
50.5	0.980	42.1	39.2
58.6	0.706	31.6	28.3
65.0	0.538	24.6	21.6
65.3	0.531	24.6	21.3
69.4	0.441	20.8	17.7
72.7	0.376	18.3	15.0
73.3	0.364	18.5	14.6
75.9	0.318	16.4	12.7
77.5	0.290	15.2	11.6
78.6	0.272	13.1	10.9
79.9	0.252	12.3	10.1
81.0	0.235	11.3	9.38
83.4	0.198	9.66	7.98
84.5	0.182	8.80	7.32
86.8	0.152	6.80	6.06
90.2	0.109	5.53	4.34
91.1	0.098	4.30	3.86
96.8	0.033	1.04	1.32
97.3	0.028	0.88	1.10
100	0	0	0

* Calculated from equation 4.

sample is brought from 0°C. to 40°C. This calculation is summarized in equation 4.

$$H_{40} = T(h/f_s - h_s) \quad (4)$$

where H_{40} is the heat capacity of the water associated with 1 g. of starch; T is the temperature change; h , h_s are the heat capacities (per degree)

of the sample and dry starch; and f_s is the fraction of starch in the sample. These values appear in column 3 of Table 3, and as the points and the broken line in Fig. 2.

The theoretical heat capacity of the water associated with 1 g. of starch, according to Robinson (8), was calculated from equation 4 by substituting for h the values in Table 2, column 3. These theoretical

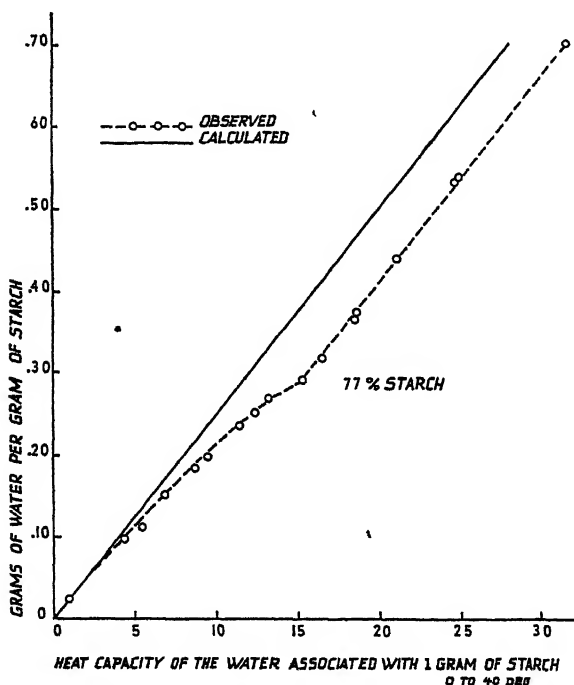


Fig. 2. Observed and calculated heat capacities of starch-water suspensions

values are given in Table 3, column 4. In Fig. 2 this theoretical relation between heat capacity and composition appears as the solid line.

All the samples listed in Table 3 are not included in Fig. 2 since the observed and theoretical lines parallel each other except in the vicinity of the 75 per cent starch samples. Consequently, only this portion of the data has been illustrated in Fig. 2.

If, as suggested by Hampton and Mennie (4), this discrepancy between the observed and theoretical heat capacities is due to the adsorption or desorption of water, it would be expected in lyophilic systems

in general; but would not appear in lyophobic systems, or in systems where the adsorption of water was small. While this point has not been extensively investigated, experiments on sand-water mixtures bear out this idea. The sand particles were relatively large and should adsorb a very small amount of water compared to such lyophilic systems as gelatin, starch, or dextrin sols. Consequently, the observed and theoretical values for heat capacities of these samples should agree closely. Good agreement is shown in Table 4.

TABLE 4

Observed and Theoretical Heat Capacities (0° to 40°C.) of Sand and Water Mixtures

Sand content	Observed heat cap	Theoretical heat cap.
<i>per cent</i>	<i>cal per gram per degree</i>	<i>cal. per gram per degree*</i>
100.0	0 176	
88.9	0 265	0.267
80.1	0.339	0.340
79.0	0 350	0.349
75.4	0 382	0.379
70.4	0.418	0.420
60.0	0.510	0.506
50.3	0.587	0.586
40.4	0.671	0.667
40.2	0.666	0.669
30.4	0.743	0.750
21.9	0.821	0.820
20.2	0.839	0.834

* Calculated from equation 3.

DISCUSSION

Hampton and Mennie (4) suggested that when the temperatures of the gelatin gels were raised or lowered, water was either desorbed or adsorbed with an accompanying gain or loss of heat. The curves in Fig. 2 strongly support this suggestion. When the temperature of starch-water systems was raised from 0° to 40°C., the sum of the heat capacities of the container, dry starch, and water was calculated to be less than the observed values. This means that more heat is absorbed by the sample than is theoretically required to raise its temperature from 0° to 40°C. Fig. 2 shows that this excess heat is constant at about 0.0875 cal. per degree for the amount of material containing 1 g. of dry starch when the samples contain more than 23 per cent water. When the samples con-

tain less than 23 per cent water, the excess heat absorbed decreases regularly with the water content.

These observations are logically explained by following Hampton and Mennie's (4) suggestion. When the temperature rises from 0° to 40°, the thermal agitation of the adsorbed water molecules is increased sufficiently to partly overcome the adsorptive forces. Consequently, a portion of the water molecules desorb. These experiments offer no evidence as to the amount of bound water desorbed by this particular temperature rise. It is obvious that the fraction desorbed would depend upon the temperature range chosen. Consequently the bound water desorbed under these conditions is simply referred to as a loosely bound fraction. The relation of this fraction to the total bound water is the subject of further investigation. The desorption of bound water, however, must be accompanied by the absorption of heat and the amount of heat absorbed for this purpose would be the difference between the curves of Fig. 2.

According to independent measurements, all the water up to 23 per cent is bound on the starch micelles. Therefore, in this sample range, the amount desorbed would increase with the amount of water present and increasing amounts of heat would be absorbed. Thus the observed and theoretical curves in Fig. 2 begin together at 0 per cent water and diverge continually to 23 per cent moisture. When the samples contain 23 per cent water, the maximum bound water has been reached, and at this point about 3.5 cal. are taken up by the sample to desorb the loosely bound fraction from 1 g. of starch. Since the maximum bound water has been reached at 23 per cent, further increments of water only add to the free water. Since the bound water content remains the same in all the remaining samples, the fraction desorbed is the same, and the amount of heat required by the desorption remains the same. In other words, from 77 per cent starch to 0 per cent starch, the observed and theoretical curves of Fig. 2 parallel each other.

From these data it seems probable that the specific heat (or heat capacity) of a system calculated by Robinson's equation 3 does not hold for lyophilic systems over any appreciable range of temperatures. It is more likely that a generalized form of equation 2 is a more adequate description, only applicable, however, to systems that have their maximum bound water content.

$$H_r = h_s f_s + h_u f_u \pm x f_s \quad (5)$$

If the adsorption of water is reversible in the system under the chosen conditions, the term αf_s will be positive or negative depending on whether the experiment was carried out with a rising or falling temperature. The term α is not a constant but depends on the temperature range used, and is determined by such lyophilic properties of the adsorbing material as: the water-binding capacity of the system, and the intensity of the water-adsorbing forces at the surface of the micelle.

The results of these measurements on starch and dextrin sols suggest the possibility of using this technique for estimating the bound water content of lyophilic systems. From the data in Figs. 1 and 2 and from the discussion above it seems evident that a break in the curves appears at the maximum bound water content. Thus a series of specific heat measurements that would include this break should indicate the maximum bound water content of the system. The method is time consuming and laborious, and would hardly be practical for any number of determinations. However, the approach is quite different from most of the present techniques and since the conditions imposed on the sample are not as drastic as in other methods, it is conceivable that it might be useful as a supplementary method in special problems. It should be noted that the break in the curve (Fig. 2) would be expected to shift with different temperature ranges, although it is quite possible that the differences might be so small that highly refined measurements would be necessary to detect them. While there is no positive assurance that this technique can be successfully applied to other lyophilic systems, experiments are in progress to determine this point.

Hampton and Mennie's (4) proposal for calculating the bound water content of lyophilic systems was based on the observation that heat capacities of gelatin systems were lower than the theoretical values, presumably due to a lower specific heat of bound water. This was apparently true over temperature ranges from well below 0° up to 25°C., but measurements made just between 0° and 25° indicated that the gelatin absorbed an excessive amount of heat. Since this complication is apparently due to the desorption of water with a rising temperature, the verification of the bound water calculation and the actual value of the specific heat of bound water depends upon the evaluation of correction terms for the heat of desorption, not only over the temperature ranges above 0°, but also over the ranges below 0°.

A possible evaluation of these correction terms is indicated by the

data on starch and dextrin sols wherein the heat of desorption was measured and calculated with some degree of certainty (Fig. 2). Therefore it would appear that an adequate correction term for Hampton and Mennie's (4) bound water calculation can be obtained for the temperature ranges above 0°C.

Similar behavior might be expected in the temperature ranges below 0°C., although there is no information available at present. It can be argued, however, that if the bound water is unfrozen below 0°, a rising temperature would similarly effect some desorption accompanied by a measurable absorption of heat. The situation may be complicated, however, by the freezing of the desorbed water and the accompanying latent heat of fusion.

Nevertheless, it appears possible that such measurements might be extended to the temperature ranges below 0°C. and, if successful, correction terms would be available to test the formula for bound water proposed by Hampton and Mennie (4).

SUMMARY

Abnormal heat capacities previously reported for gelatin and gum arabic were also found in mixtures of raw starch and water and in dextrin sols, but were not found in sand-water mixtures. The results strongly suggest that such behavior may be generally found in lyophilic systems.

Explanation of the data bears out the suggestion of Hampton and Mennie (4) that when the temperature of the system is raised, a portion of the bound water is desorbed. This process is accompanied by the absorption of heat and this heat effect is superimposed on the heat capacities of the constituents of the sample.

Robinson's (8) equation for the specific heat of biological systems was found invalid for starch and dextrin systems and probably does not hold for most lyophilic systems. Equation 2 describing the specific heat of starch systems was derived from the experimental data and a general equation 5 for lyophilic systems was proposed.

The measurements of heat capacity indicate that under the conditions of the experiment all the water up to 23 per cent is adsorbed by the starch, but a portion of it is so loosely held that it is desorbed when the temperature rises from 0° to 40°. This value for bound water is in good agreement with those obtained by other methods.

Hampton and Mennie's (4) equation for calculating the bound water from specific heat data cannot be applied in its present form. However,

a correction term for temperature ranges above 0° has been calculated from the data on starch and the possibility of obtaining a correction term for temperature ranges below 0° has been indicated.

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The Metabolism of Crotonic Acid¹

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During studies on pyruvate oxidation (1) we became interested in various aspects of fatty acid oxidation, and were aware that little definite knowledge is available about the intermediaries between the fatty acid and its first established oxidation product, acetoacetic acid. A sequence, desaturation, hydration and dehydrogenation has often been assumed as analogous to the sequence, succinic \rightarrow fumaric \rightarrow malic \rightarrow oxaloacetic acid. In the case of the oxidation of butyric acid in animal tissues, such a scheme seemed reasonably supported by the presence of systems oxidizing both, crotonic and β -hydroxybutyric acids to acetoacetic acid. However, Friedmann and Maase (2) found as early as 1913 that crotonate could be converted to β -hydroxybutyrate only when oxygen was present and when acetoacetate was produced at the same time. Jowett and Quastel (3) supported Friedmann and Maase's view that the hydroxybutyrate was formed indirectly from butyrate and crotonate. The fact then that an unsaturated fatty acid could be oxidized to the corresponding keto-acid without intermediation of hydroxyacid, seemed to us surprising, and if true, suggested an unusual type of biological oxidation. It was, therefore, decided to study the metabolism of crotonic acid in some detail.

The study reported here was undertaken some time ago but due to adverse conditions remained in a preliminary state. Nevertheless it is presented now because it is believed that the question touched upon is of general importance. In recent work from this laboratory by Barker and one of us (L.) (4), a very similar problem was encountered

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regarding the interrelation of propionic, lactic, and pyruvic acids in propionic acid fermentation. Related problems concerning the metabolism of the two pairs of acids, fumaric-malic and aconitic-citric will be discussed later in this paper.

METHODS

Estimation of Crotonic Acid. Very satisfactory results were obtained with the bromometric method down to less than 0.1 mg. of crotonic acid

TABLE I
Bromometric Titration of Crotonic Acid
1 mg. crotonic acid = 2.33 ml. *N*/100 bromine

Added	<i>N</i> /100 bromine used	Found
mg.	ml.	mg.
0.043	0.11	0.047
	0.10	0.043
	0.08	0.034
0.086	0.20	0.086
	0.21	0.090
	0.16	0.070
0.43	1.08	0.47
	1.00	0.43
0.86	2.00	0.86
	1.94	0.84

when loss of bromine through evaporation or contact with organic material was carefully avoided. We used as reaction vessels 25 ml. volumetric flasks with well fitting glass stoppers.

The only substance found to interfere in our experiments was acetoacetic acid. No reaction however was found to take place between acetone and bromine under our conditions. Therefore acetoacetic acid could be conveniently destroyed by heating with strong acid.

Procedure. 1-2 ml. trichloroacetic acid filtrate were pipetted into a 25 ml. volumetric flask and 2 ml. *N*/1 hydrochloric acid was added. The mixture was heated on a boiling water bath for 20 minutes and

carefully cooled to somewhat below room temperature. Then 3 ml. (or more if needed) of approximately $N/100$ bromine water were added and the flask stoppered immediately. The bromine solution had to be prepared before the experiment by dilution from a concentrated stock solution. Blanks were titrated at the start and at the end, of every series. After a reaction time of 15 minutes a few crystals of potassium iodide were added, and the liberated iodine titrated with $N/100$ thio-sulfate, 1 ml. of which corresponded to 0.43 mg. of crotonic acid. Results from the titration of known amounts of crotonic acid are given in Table I.

Acetoacetic acid was determined manometrically as described by Jowett and Quastel (3).

Oxygen consumption was measured with the Warburg technique using open manometers and conical or square vessels of 16 ml. volume.

EXPERIMENTAL

Experiments with Slices. The tissues were sliced with a razor and the slices suspended in Ringer's solution before transference to the vessels. 20 to 30 mg. dry weight of tissue were used per vessel.

The medium was Ringer's solution containing $M/100$ phosphate of pH 7.5 and $M/100 - M/50$ substrate. In most experiments two separate vessels were used for the determination of crotonate and acetoacetate respectively. After the experiment the sodium hydroxide was removed carefully from both vessels. The tissue slices were lifted out with a hooked glass needle and the acetoacetate determined in one vessel. Trichloroacetic acid was added to the other and the crotonic acid determined bromometrically in the filtrate as described above.

Preparation of Tissue Extracts. We followed grossly the procedure of Kalckar (5). The cooled organs were hashed and finely ground with sand in a carefully cooled mortar to which an equal volume of $M/20$ phosphate buffer of pH 7.4 was slowly added. The suspension was centrifuged for two minutes at low speed.

Experiments with Tissue Slices. With slices of rat liver tissue, Table II shows the *large aerobic* disappearance of crotonate in contrast to a *negligible anaerobic* disappearance as shown in Table III. Aerobically half of the crotonate is accounted for by acetoacetate while most of the other half is probably β -hydroxybutyrate (2, 3) which we did not determine because crotonate interferes with the chemical determination

of hydroxybutyrate. Furthermore it should be emphasized that the hydroxybutyrate neither aerobically nor anaerobically gave rise to

TABLE II

Respiration of Crotonate by Rat Liver Slices (from 24 h. fasted rats)
Simultaneous determination of disappearance of crotonate, oxygen
consumption and acetoacetate formation

$$Q = \frac{\mu\text{l.}}{\text{mg. dry tissue} \times \text{hours}} \quad \text{One millimol} = 22,400 \mu\text{l.}$$

Substrate	$-Q_{O_2}$	$Q_{\text{acno.}}$	$-Q_{\text{croton.}}$	$\frac{Q_{\text{acno.}}}{Q_{\text{croton.}}}$
None	6.6			
M/100 crotonate	9.8	3.4	6.6	0.52
None.	6.0			
M/100 crotonate	7.5	3.1	9.0	0.35
None	8.0	0.6		
M/100 crotonate	9.6	4.3	7.1	0.61
M/100 crotonate.....	6.6	3.0	7.1	0.42
M/50 crotonate.....	8.9	4.8	9.5	0.47
None...	8.0			
M/100 butyrate	10.0	4.2	0	
M/50 d,l- β -hydroxybutyrate.. . . .	7.2	2.1	0	

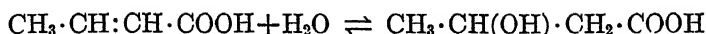
TABLE III

Anaerobic Metabolism of Crotonate and β -Hydroxybutyrate with Rat Tissue Slices

Substrate	$Q_{\text{acno.}}$	$Q_{\text{croton.}}$	Organ
M/100 crotonate...	0	-0.12	Liver
M/100 crotonate.	0	-0.09	Liver
M/100 crotonate.....	0	-0.11	Liver
M/100 hydroxybutyrate.		0	Liver
M/100 crotonate.....	0	-0.5	Kidney

bromine binding substance, i.e., crotonate. Therefore, since anaerobically, crotonate did not disappear to form β -hydroxybutyrate and since

β -hydroxybutyrate did not yield any crotonate, the possibility of an equilibrium,



was excluded.

With rat kidney slices crotonate is metabolized anaerobically as shown in the lowest section of Table III and appears more clearly in experiments with kidney extracts.

Experiments with Liver and Kidney Extracts. Extracts from liver (rat) and kidney (rabbit) which respire butyric and crotonic acid were prepared by homogenization with an equal volume of phosphate buffer and superficial centrifugation as described above. The oxidation of

TABLE IV

Respiration of Crotonate and Butyrate by Rat Liver Extracts

Acetoacetate formation by 2 ml. extract one hour incubation at 37.5°
 $\mu\text{l. acetoacetate}^1$

Substrate None	M/100 crotonate	M/50 butyrate
36	165	97
51	227	54
69	118	174
112	165	353
54	64	65
50	62	81

* In extracts, acetoacetate was determined after deproteinization with cooled trichloroacetic acid or with 1.5% uranyl acetate.

butyrate in homogenized liver was previously described by Leloir and Muñoz (6). In aerobic experiments with liver only acetoacetate formation was followed (Table IV). In two experiments more acetoacetate was formed from crotonate than from butyrate; however, in the majority butyrate was the better substrate. The activity of such extracts was rather variable. Anaerobically (Table V), the result with liver extracts duplicates that with liver slices. No crotonate disappeared and none was formed from β -hydroxybutyrate. Acrylate, likewise, was stable with liver extracts under anaerobic conditions.

A new observation is the anaerobic metabolism of crotonate with kidney. The extract experiments (Table V) show a fairly large utilization. Up to 45 per cent of the total added amount of crotonate was

metabolized at the end of two hours. No acetoacetate was formed. As already mentioned crotonate interferes with the iodometric determination of hydroxybutyrate. A rather crude determination was carried out by adding afterwards the same amount of crotonate to the control. Such determinations showed a decrease of total iodine consuming substance in the distillate from bichromate, instead of an increase as would

TABLE V

Anaerobic Metabolism of Crotonate, β -Hydroxybutyrate and Acrylate with Extracts of Rat Liver and Rabbit Kidney

Substrate	Extract	μ l. crotonate				μ l. acetoacetate formed
		Added	Found after 2 h. incubation at 37.5°	Disappeared	Formed	
Crotonate.....	Liver	423	390	33	0	
Hydroxybutyrate.....	Liver	896				
Acrylate.....	Liver	870	840	30		
Crotonate.....	Kidney	390	215	175		18-14
Crotonate.....	Kidney	446	366	80		11-7
Crotonate.....	Kidney	446	310	136		.

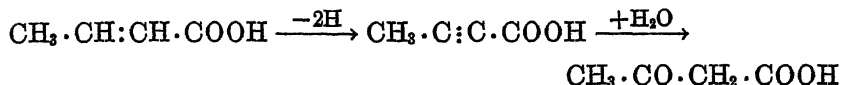
TABLE VI

Influence of the Addition of Tetrolate on the Respiration and Acetoacetate Formation of Rat Tissue Slices

Substrate	Q_{O_2}	$Q_{acac.}$	Tissue
None.....	14.5	0.3	Kidney
M/50 tetrolate.....	14.3	0.25	
None.....	6.1	0.7	Liver
M/50 tetrolate.....	5.2	0.6	

be expected from hydroxybutyrate formation. These determinations reasonably exclude the possibility that the breakdown product of crotonate in the kidney is β -hydroxybutyrate and further evidence is established that in neither the kidney nor the liver is there an equilibration between crotonic and β -hydroxybutyric acids. We consider it, therefore, most probable that the anaerobic disappearance of crotonate in the kidney is due to reduction.

Experiments with Tetrollic Acid. It was considered worthwhile to check upon the possibility that crotonic acid may be first further de-saturated to the corresponding acetylene derivative, tetrollic acid. Tetrollic acid seems to add water fairly easily to form acetoacetic acid (7), suggesting then the following sequence of reactions:



We therefore prepared tetrollic acid according to Feist (8). As shown in Table VI, the assay of tetrolate with liver and with kidney slices gave negative results, both with respect to oxygen consumption and acetoacetate formation. Thus, tetrollic acid is not likely to be an intermediate in fatty acid oxidation (Table VI).

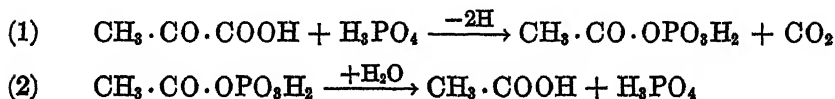
DISCUSSION

The reported experiments seem definitely to exclude a straightforward hydration of a fumarase or aconitase type as an intermediary step in the oxidation of crotonic to acetoacetic acid in animal tissues. An analogous situation seems to exist in the butanol-acetone fermentation. Here, according to Bernhauer and Kuerschner (9), crotonic acid is fermented to acetone and butanol; but Johnson, Peterson and Fred (10) found that β -hydroxybutyric acid was neither formed nor fermented.

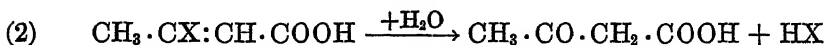
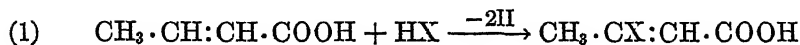
The question then arises if the overall reaction:



may in fact represent not dehydrogenation, but an oxygenation as proposed by Jowett and Quastel (3). However, other explanations are possible. It was shown recently in the case of carbonyl compounds that instead of water, phosphoric acid may be added before, and rapidly removed after dehydrogenation (11). In *Bacterium delbrueckii* the dehydrogenation of pyruvic to acetic and carbonic acids was shown to occur in two phases:



A similar mechanism may be suggested for crotonic acid oxidation:



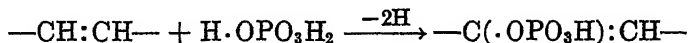
X may be phosphate or any anion, it also may be an enzyme. It should be noted that when X is phosphate the primary dehydrogenation product is an enol-phosphate.

In spite of the existence of fumarase and of aconitase which equilibrate the unsaturated polycarboxylic acids with the corresponding hydroxy-acids, doubts have been raised whether the routes leading over malic or citric acid respectively are the main routes of degradation. Thus, for various reasons, one of us (L.) (12) suggested a mechanism for the breakdown of fumaric acid as analogous to the one outlined above for crotonic acid. Elliott (13) commented recently on differences in the effects of malate and fumarate on respiration.

Wood and Werkman (14), Evans and Slotin (15) and Starc et al. (16) propose a modification of Krebs' citric acid cycle with the omission of citric acid. The elimination of the symmetrical citric acid is considered necessary to explain the assymmetrical position of the fixed CO_2 in ketoglutaric acid. Quoting Werkman and Wood (17):

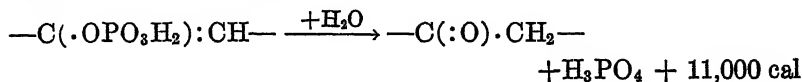
"It is recognized that most tissues contain aconitase which would induce formation of citrate but as a working hypothesis the skeleton of the original cycle might as well be retained until information is available which dictates the proper change. It is probable that phosphorylated intermediate compounds are involved."

Apparently ambiguities are encountered in many cases where the biological oxidation of the carbon to carbon double bond is supposed to be accomplished by a two step reaction involving primary hydration of the double bond with subsequent dehydrogenation of hydroxy- to keto-acid. As a working hypothesis it is proposed that primarily a dehydrogenation associated with phosphorylation occurs; formulated as follows:



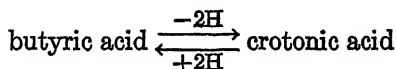
Through this reaction scheme a means could be provided by which through accumulation in the enol-phosphate, bond energy is prevented

from dissipation. This energy can be drawn off in the final reaction leading to the keto compound:



II

Some of the results reported here have a bearing on the problem of desaturation as an intermediary step in fatty acid oxidation. The comparative ease with which crotonic acid is metabolized, described earlier by other workers, was confirmed here. However, an accumulation of crotonic acid in the course of butyric acid oxidation was not observed (bottom of Table II). Nevertheless indication for the existence of a reversible oxido-reduction system catalysing the reaction,



is seen in the experiments with kidney, where in addition to the well known oxidation of butyric acid, an apparent reduction of crotonic acid was observed. Reduction of crotonic acid in bacteria is indicated by the derivation of butanol from crotonic acid (9).

SUMMARY

1. A micro method for determination of crotonic acid is described.
2. With rat liver slices in *oxygen*, the following Q-values were found: Q(crotonate) -8 , Q(acetoacetate) 3.5 , Q(extra O_2) -2 .
3. *Anaerobically*, crotonate was not attacked by liver slices or extracts, and no conversion of β -hydroxybutyrate to crotonate was found. This is regarded as proof that an equilibration between crotonic and β -hydroxybutyric acid does *not* take place, and that β -hydroxybutyrate is not a precursor but a reduction product of acetoacetate.
4. An anaerobic breakdown of crotonate, presumably by reduction, was found with kidney preparations.
5. The mechanism of oxidation of —CH:CH— to $\text{—CO}\cdot\text{CH}_2\text{—}$ in compounds as crotonic, fumaric and aconitic acid is discussed.

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The Inverse Ratio between Fluoride in Food and Drink and Dental Caries¹

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The senior author and his students (19) showed that fluorapatite is insoluble in saliva at pH 5.5 to 7 and that feeding fluorine to rats is beneficial to their teeth (9). This idea was kept in mind—we quote: “The teeth require calcium, fluorine and phosphate” (8), “the enamel of the teeth is much harder than calcium phosphate and is probably 20% fluorapatite” (10) and “up to a certain point the greater the fluorine content of the enamel the greater its hardness” (11).

Armstrong (1) showed that the enamel of sound human teeth contained more fluorine than the enamel of carious teeth, and Hodge and collaborators (7) on the basis of Armstrong's analyses of rats' teeth came to similar conclusions for rats (attributing the source of fluorine to be casein). Cox and collaborators (3) found that fluorine fed to mother rats inhibited caries in the offspring. Dean, Jay, Arnold and Elvove (6) showed that children who for several years after birth drank water of high fluorine content but for the subsequent 12 years drank “fluorine-free” water had less dental caries than those who drank the same “fluorine-free” water from birth. All these papers indicate that fluorine acts as a building-stone of the enamel.

On the contrary, the view that fluorine acts by inhibiting mouth bacteria is supported by the following: the other surveys by Dean and collaborators (5) who showed that fluoride in drinking water lessened the number of *B. acidophilus* in the saliva; the experiments of Miller (16) who showed that iodoacetic acid acted like fluoride in inhibiting caries in rat molars with fully formed enamel; and Arnold and McClure (2) who

¹ Aided by a grant from the Penrose Fund of the American Philosophical Society.

showed that injected fluoride did not protect against caries. In considering this evidence the following points might be borne in mind: the fact that fluoride protects erupted teeth is no proof that it does not enter the crystal lattice of the enamel since isotope studies as well as total analyses (18) have shown that elements enter the enamel after it is formed; the results of Arnold and McClure are at variance with previous work by McClure (15) as well as that of McClendon and Foster (13, 14) in that their experiments "show little, if any, significant reduction in dental caries" on adding 10 parts per million of fluorine to the rats' drinking water, which is 10 times the concentration shown to be effective in reducing human caries.

Because the careless addition of fluoride to drinking water is dangerous and in order to determine whether fluorine might act in other than bacteria-inhibiting ways we determined the fluorine content of foodstuff from different localities and soil conditions with a view of comparing the data with the Draft Board data on dental caries compiled by Davenport and Love (4). It now appears evident, however, that the Draft Board considered filled teeth as sound teeth and Davenport and Love record only the geographical distribution of unfilled caries (which probably varies inversely as the geographic distribution of dentists). We are therefore limited to states which have been surveyed in the U. S. Public Health Service Bulletin 226 (20). We will limit the data in this paper to the fluorine content of milk and since two years were required for the analyses given, we are publishing the results without obtaining milk from more localities.

These data on caries show how important it is to have the data comparable as to age, sex and method of scoring. Since not all of the counties from which we obtained milk are surveyed for caries and since it would be hazardous to mix these data with data from other sources, we have adopted the plan of comparing averages of wide areas. Perhaps the best division of the United States is the 40th parallel of latitude. Mills (17) compiled the dental caries studies of the U. S. Public Health Service according to latitude. The average number of caries per 100 children of 12 to 14 years in cities south of the 40th parallel was 327 and north of the parallel 473 whereas the average micrograms of fluorine per 10 grams of milk solids of states south of the 40th parallel is given in Table I as 17 and north of the 40th parallel as 12.

Milk from the part of Ontario adjacent to New York may be taken as

TABLE I
Micrograms of Fluorine per 10 g. of Milk Solids

States S of 40°	County	Town	Winter spray skim	Winter evap.	Spring spray	Spring evap.	Fresh	Av.	State av.	Av. S. of 40°.
Tex.	Deaf Smith McLennon	Hereford Waco	9	36	19		34	34 21	28	
Miss.	Noxubee	Macon	8			9		9	9	
Tenn.	Lincoln Marshall	Fayetteville Lewisburg	8	12	15	10		12 11	12	
Ariz.	Maricopa	Tempe		39		13		26	26	
Kan.	Bourbon	Ft. Scott		8		11		10	10	17
Wash.	Lewis	Chehalis		12		13		13	13	
Neb.	Lancaster Douglas	Lincoln Waterloo	10 8		14 16			12 12	12	
Wis.	Waupaka Waupaka Green Columbia Fond du Lac	New London Clintonville Browntown Columbus Fond du Lac	6 9 5 6	20	9 13 20 12	9		15 8 11 13 9	11	
Mich.	Shiawassee Ogemaw Gratiot	Owosso West Branch Perrinton	8 6 4		11 8*	9		10 7 8	8	
Iowa	Woodbury	Sioux City		15		13		14	14	
Penna.	Erie	Union City	4		16			10	10	
N. Y.	Allegheney Jefferson Chenango Cattaraugus Courtland Wyoming Wyoming	Belmont Evans Mills Bainbridge Delevan Cincinnatus Arcade Perry	5 5 4 8 6 5 5		19 35 13 18 16 44 23 [†]			12 20 9 13 11 25 14	15	
Vt.	Chittenden	Richmond	5		29			17	17	12 [‡]

* Whole milk, the other samples of this column are skim milk.

† Average N. of 40°.

additional evidence of the fluorine of the New York region and was as follows (Table II).

In Table I all the spray dried milk was skim milk except the spring spray from Perry, N. Y., and West Branch, Mich. The evaporated milk contained the butter fat and averaged higher in fluorine than the skim milk. We began some determinations on the distribution of fluorine in milk but reserve them for a later paper.

The selection of the age group of the children at 12 to 14 years was to simplify the statistics. In general, caries in this age group varied proportionally to caries in other age groups. The selection of city children is justified by the fact that most of the milk was drunk by city children. This milk was transported (10 miles) and so was some of the feed for

TABLE II
Milk from Ontario
Micrograms of fluorine per 10 g. of milk solid

Town	Winter spray skim	Spring spray skim	Average	Ontario average
Belmont	12	14	13	
Tillsonburg.....	6		6	
Ottawa.....	3	24	14	
Princeton.....	5	16	11	
Maintland.....	9	12	11	11

This average of 11 micrograms of fluorine per 10 g. of skim milk solids is close to the figure of 12, the average of United States north of the 40th parallel.

the cows. Perhaps the spring feed was to a larger proportion local. The higher fluorine content of the spring milk may have been due to the higher proportion of grass to grain or concentrate.

The analyses given in Tables I and II were done in duplicate or triplicate by the method of McClendon and Foster (12) which involves burning the sample in a closed platinum combustion tube, distillation with superheated steam and analysis at constant pH. Owing to the time involved relatively few samples could be analyzed but it is thought better to make a few accurate analyses than numerous analyses of questionable accuracy. Since the method is not accurate for less than a microgram of fluorine, fractions of a microgram less than 0.5 have been omitted and those of 0.5 or greater have been considered as 1 microgram.

The use of political divisions (states) does not lend itself to statistical

TABLE III

Relation of Carious Teeth per 100 Children and Micrograms Fluorine per 10 cc. Milk to Latitude

Latitude	Carious teeth per 100 children 12-14 yrs.	Town and State	γ F per 10 g. milk solids	Average
<i>degrees</i>				
25-32.9	271	Waco, Tex.	21	21
33-34.9	296	Hereford, Tex.	34	23
33-34.9	296	Macon, Miss.	9	
33-34.9	296	Tempe, Ariz.	26	
35-35.9	299	Fayetteville, Tenn.	12	12
35-35.9	299	Lewisburg, Tenn.	11	
37-37.9	380	Ft. Scott, Kan.	10	10
40-40.9	476	Lincoln, Neb.	12	12
41-41.9	447	Waterloo, Neb.	12	12
42-42.9	467	Browntown, Wis.	11	13
42-42.9	467	Sioux City, Ia.	14	
42-42.9	467	Union City, Pa.	10	
42-42.9	467	Arcade, N. Y.	25	
42-42.9	467	Bainbridge, N. Y.	9	
42-42.9	467	Belmont, N. Y.	12	
42-42.9	467	Cincinnati, N. Y.	11	
42-42.9	467	Delevan, N. Y.	13	
42-42.9	467	Perry, N. Y.	14	
42-42.9	467	Belmont, Ont.	13	
42-42.9	467	Tillsonburg, Ont.	6	
43-43.9	527	Columbus, Wis.	13	10
43-43.9	527	Fon du Lac, Wis.	9	
43-43.9	527	Owosso, Mich.	10	
43-43.9	527	Perrinton, Mich.	8	
43-43.9	527	Princeton, Ont.	11	
44-44.9	465	Clintonville, Wis.	8	13
44-44.9	465	New London, Wis.	15	
44-44.9	465	West Branch, Mich.	7	
44-44.9	465	Evans Mills, N. Y.	20	
44-44.9	465	Richmond, Vt.	17	
44-44.9	465	Maintland, Ont.	11	
45-45.9	482	Ottawa, Ont.	14	14
46-46.9	587	Chehalis, Wash.	13	13

study and since Mills has divided the country by parallels of latitude, in order to find the correlation coefficient it is necessary to arrange the data as in Table III.

The data may then be collected as in Table IV.

With this arrangement the correlation coefficient of caries to fluorine is -0.37 .

TABLE IV

Number of Towns with Micrograms Fluorine per 10 cc. Milk as on Abscissa in Latitudes with Carious Teeth per Child as on Ordinate

Carious teeth per child	Micrograms fluorine per 10 cc milk					
	5-9	10-14	15-19	20-24	25-29	30-34
5-6	2	4				
4-5	4	12	2	1	1	
3-4		1				
2-3	1	2			1	1

SUMMARY

Dental caries in city school children varies inversely with the fluorine content of cows' milk. The correlation coefficient is -0.37 .

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Carbon Monoxide Inhibition of Nitrogen Fixation by *Azotobacter*¹

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Previous studies established that extremely small quantities of carbon monoxide inhibit symbiotic nitrogen fixation by inoculated red clover plants (2). Preliminary tests by Wyss (6) indicated that a similar inhibition occurs with *Azotobacter vinelandii*. As this free-living nitrogen-fixer possesses many advantages for the detailed investigation of the inhibition, our recent investigations have been made with it employing both macro total nitrogen and microrespiration techniques. This report summarizes the findings of the macro experiments.

METHODS

Cultures of *Azotobacter vinelandii* were maintained by daily transfer in a liquid medium consisting of Burk's salts plus 2% sucrose and 0.25% agar (3). Four bottles (60 ml.) of a 24 hour culture were used to inoculate a liter of the same medium which was incubated at 30°C. for 12 hours, then 35 ml. transferred aseptically to sterile liter serum bottles. Details of the method for controlling the pO_2 during the experiment were described by Wyss and Wilson (7). Total nitrogen was estimated by a semimicro method, the cells being precipitated with colloidal iron in the combined nitrogen series. Tests demonstrated that added combined nitrogen (300 p.p.m.) was quantitatively recovered in the cells and supernatant fluid. Other details of the technique such as preparing the carbon monoxide and the statistical method for estimating the specific rate constant of fixation, the k value, are described in our earlier papers.

AIR SERIES

The data of Figs. 1 and 2 show that fixation of atmospheric nitrogen by *A. vinelandii* is definitely inhibited by as little as 0.2% CO in the air, but uptake of NH_4NO_3 -N is unaffected even by 0.5% CO. Further experiments, which included ammonium phosphate and sodium nitrate

¹ This research was supported in part by grants from the Rockefeller Foundation and from the Wisconsin Alumni Research Foundation.

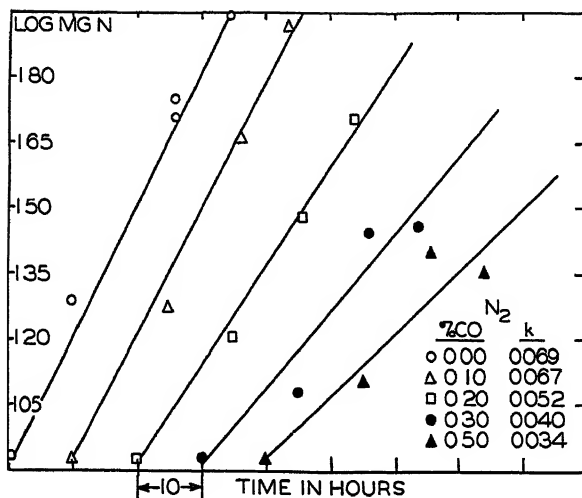


FIG. 1. Carbon monoxide inhibition of nitrogen fixation by *Azotobacter vinelandii* in air. (In the figures a sliding scale is used for the abscissa so that the individual lines are distinct; zero for each line is represented by the initial point.)

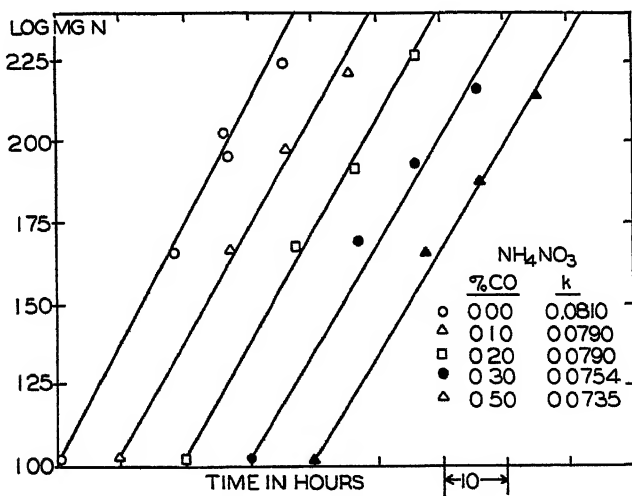


FIG. 2. Assimilation of $\text{NH}_4\text{NO}_3\text{-N}$ by *Azotobacter vinelandii* in presence of air containing CO

TABLE 1

Effect of CO on Assimilation of Free and Combined Nitrogen by Azotobacter vinelandii (Air Experiments)

Expt.	% CO in atm.	Source of nitrogen	Final nitrogen content		k value	Relative k value
			Total*	Relative		
1	0.00	N ₂	14.4	100	0.0810	100
	0.05		13.4	93	0.0791	98
	0.10		13.4	93	0.0765	94
	0.20		11.3	79	0.0713	88
	0.40		3.5	24	0.044	54
	0.00	NH ₄ NO ₃	11.5	100	0.0800	100
	0.05		11.7	102	0.0790	99
	0.10		10.9	95	0.0765	96
	0.20		11.6	101	0.0753	94
	0.40		10.6	92	0.0745	93
2	0.00	N ₂	4.1	100	0.0828	100
	0.10		4.3	105	0.0805	97
	0.20		3.3	79	0.0690	83
	0.30		1.3	31	0.0381	47
	0.50		1.3	31	0.0366	44
	0.00	NH ₄ NO ₃	9.6	100	0.0775	100
	0.10		9.7	102	0.0755	98
	0.20		8.3	87	0.0696	90
	0.30		8.4	88	0.0712	92
	0.50		6.3	65	0.0605	80
3	0.00	N ₂	11.6	100	0.0867	100
	0.05		10.8	94	0.0834	102
	0.10		7.7	67	0.0786	91
	0.20		7.0	61	0.0721	83
	0.40		3.4	29	0.0503	58
4	0.00	NaNO ₃	19.7	100	0.0760	100
	0.20		19.9	101	0.0795	104
	0.40		18.4	94	0.0766	101
	0.00	(NH ₄) ₂ HPO ₄	10.8	100	0.0783	100
	0.20		10.6	98	0.0800	102
	0.40		7.7	72	0.0693	89

TABLE 1—*Concluded*

Expt	% CO in atm	Source of nitrogen	Final nitrogen content		k value	Relative k value
			Total*	Relative		
5	0.00	NaNO ₃	14.3	100	0.0723	100
	0.20		15.0	104	0.0694	96
	0.40		12.0	84	0.0624	86
	0.00	(NH ₄) ₂ HPO ₄	13.9	100	0.0752	100
	0.20		12.8	93	0.0715	95
	0.40		10.5	76	0.0658	87

* Values in all tables mg. per 100 ml.

Expt.		Initial N content	Time in hours	Significant difference in k
1	N ₂	0.87	36	0.0127
	NH ₄ NO ₃	0.71		0.0103
2	N ₂	0.45	28	0.0112
	NH ₄ NO ₃	1.16		0.0076
3	N ₂	0.54	36	0.013
4	NaNO ₃	1.05	39	0.022
	(NH ₄) ₂ HPO ₄	0.64		0.022
5	NaNO ₃	1.49	34	0.026
	(NH ₄) ₂ HPO ₄	1.36		0.026

are given in Table 1. Occasionally a slight inhibition of the uptake of combined nitrogen at the higher levels of CO occurred, but the response was inconsistent and when obtained was quantitatively much less than that with N₂. Results with asparagine as the source of nitrogen were similar to those shown in Fig. 1, suggesting that fixation predominates over assimilation of asparagine-N when air is used. Studies with the microrespiration technique and with isotopic nitrogen to be described in other reports confirmed this suggestion not only for asparagine but for other organic forms as well as for nitrate and nitrite.

To avoid this difficulty, we substituted N₂-free gas mixtures for air. A helium-oxygen mixture was first tried since the organism possesses an active hydrogenase, and it was thought that use of hydrogen might

introduce a complicating factor. Tank helium, however, contains about 2% nitrogen gas, and even at this low pN_2 , fixation predominates over assimilation of many forms of combined nitrogen (5). Further studies by the microrespiration method with a purified source of helium established that uptake of combined nitrogen by *A. vinelandii* in a H_2 - O_2 mixture was the same as in a He - O_2 mixture. The preliminary studies also indicated that about 0.6% CO represented the critical level of the inhibitor, since this concentration almost completely suppressed fixation of N_2 but had little effect on assimilation of NH_3 -N. In the remaining experiments with combined nitrogen, therefore, a gas mixture of 80% H_2 and 20% O_2 with and without 0.6% CO was used.

HYDROGEN-OXYGEN SERIES

Results from typical experiments with various forms of nitrogen are summarized in Table 2. No outstanding inhibition was noted with any source of combined nitrogen, but the rate of assimilation of these differed markedly. Urea is an excellent source, asparagine and nitrate less so, glutamate very poor, and nitrous oxide is probably not attacked. In Fig. 3 the k value for the uptake of urea is 0.113 and for asparagine, 0.046. The k value for glutamate was not calculated as its uptake was slow and did not remain logarithmic with time. The conclusions regarding non-inhibition by CO based on the total nitrogen data of Table 2 are confirmed by these rate experiments.

Experiments with inorganic forms of nitrogen are complicated by the fact that the pH is frequently altered during the course of the experiment because of selective utilization of one ion. To determine the magnitude of such changes, we carried out the experiments reported in Table 3 in which the pH was estimated with the glass electrode at each harvest. No great change in pH was observed when NH_4NO_3 was the nitrogen source, but with ammonium phosphate, the medium became so acid that growth practically ceased after 20 hours. Similar results have been obtained with the chloride and sulfate, but ammonium acetate serves as an excellent source of NH_3 -N. The organic radical appears to be utilized somewhat more rapidly than NH_4 so that a slight, but not unfavorable rise in the pH occurs. This increase in pH may explain why ammonium acetate appeared to be somewhat superior to ammonium nitrate in these trials. It may be related also to the differential utilization of the NH_4 and NO_3 ions to be discussed later; it should be men-

TABLE 2
Assimilation of Free and Combined Nitrogen by *Azotobacter vinelandii* in Presence and Absence of CO (Hydrogen-Oxygen Experiments)

Source of nitrogen	Time	Control	+0.6% CO
	hours		
N_2^*	20	6.95	1.65
	32	21.5	5.8
$(NH_4)_2HPO_4$	20	7.15	5.45†
	32	7.4	6.2†
$NaNO_3$	20	2.9	1.7
	32	9.8	11.75
N_2O^\dagger	32	0.65	0.5
	72	1.2	0.6
Urea	20	9.0	11.55
	32	22.6	23.45
Asparagine	32	3.55	4.25
	48	12.3	15.3
Glutamate	32	1.3	1.3
	72	2.45	3.4

* Air, all others (except N_2O) were kept in an atmosphere of 80% H_2 and 20% O_2 . Combined nitrogen—300 p.p.m. Initial harvest (0 hours)—0.47 mg./100 ml.

† Atmosphere of 75% H_2 , 20% O_2 , 5% N_2O .

‡ Low values probably arise from change in pH. See Table 3.

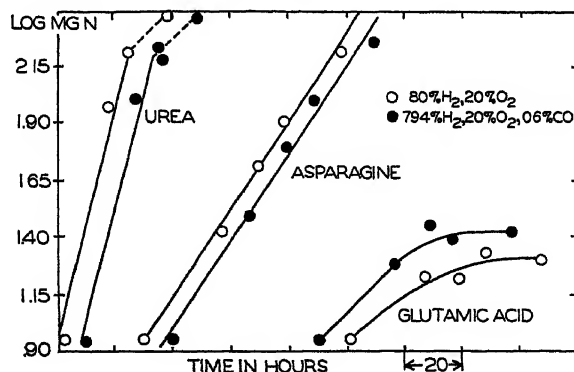


FIG. 3. Assimilation of organic forms of nitrogen by *Azotobacter vinelandii* in presence of CO

TABLE 3

Effect of Source of Combined Nitrogen on Rate of Uptake and pH

Source of nitrogen	Harvest			
	I	II	III	IV
<i>Experiment 6:</i>				
NH ₄ NO ₃ †.....	3.9	8.7	17.0	22.75
pH.....	6.77	7.18	7.12	6.97
NH ₄ Acetate.....	7.0	16.4	21.6	22.8
pH.....	7.28	7.20	6.93	6.90
(NH ₄) ₂ HPO ₄	5.75	11.15	12.3	12.8
pH.....	6.38	5.98	5.52	5.25
KNO ₃	1.3	2.55	3.4	4.65
pH.....	6.68	6.93	6.88	6.95
<i>Experiment 7:</i>				
NH ₄ NO ₃	1.45	6.55	14.1	20.75
pH.....	6.90	6.93	7.12	6.98
NH ₄ Acetate.....	2.65	9.45	17.45	22.9
pH.....	7.68	7.40	7.23	6.94
KNO ₃	0.9	2.45	6.7	8.95
pH.....	6.90	6.90	7.03	7.09
KNO ₃ + NH ₄ Ac*.....	1.45	4.7	8.8	12.8
pH.....	6.98	7.00	7.24	7.24

† Values opposite source of nitrogen are total N in mg./100 ml.

* 295 p.p.m. KNO₃-N + 5 p.p.m. NH₄ Acetate; all others 300 p.p.m. of indicated source of nitrogen.

Experiment	Initial nitrogen	Harvest			
		I	II	III	IV
		hours	hours	hours	hours
6	0.65	13	19	25	31
7	0.50	9	16	22	27

Initial pH in all cultures—7.1.

tioned, however, that the superiority of the acetate was not always observed in other experiments.

It is evident from the data in Table 3 that uptake of nitrate as judged

by total nitrogen in the cells at a given harvest is much slower than uptake of the ammonium ion. Since little change in pH occurred, this apparently is not a factor. Experiments with the nitrate of calcium, potassium and sodium verified this conclusion. The k value for uptake of nitrate, however, was frequently not much less than that for uptake of ammonium ion as is illustrated by the data in Fig. 4. Although the total nitrate-nitrogen assimilated was only about 12.5 mg./100 ml. as compared with 23.5 mg./100 ml. of ammonium-nitrogen, the k values for the different sources were: NH_4 , 0.131; NO_3 , 0.120; NO_2 , 0.085; aspartate, 0.074

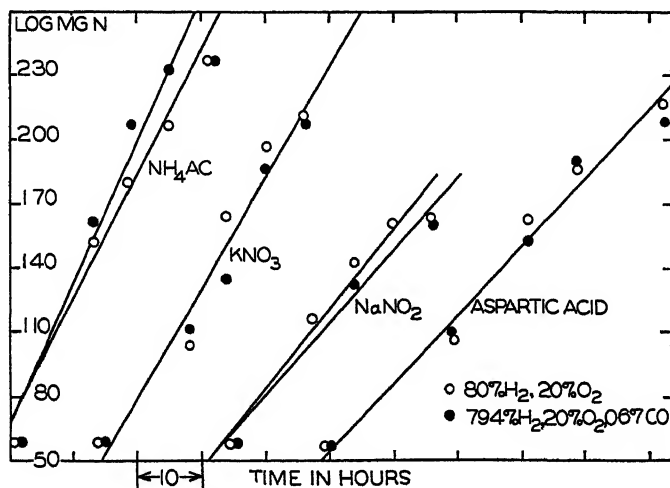


FIG. 4. Assimilation of various forms of nitrogen by *Azotobacter vinelandii* in presence and absence of CO

The toxic effect of nitrite may be a factor in the low value for this source even though only 50 p.p.m. $\text{NaNO}_2\text{-N}$ were used. In microrespiration experiments in which nitrogen was supplied at 30 p.p.m. the k values for assimilation of nitrate and nitrite were the same and consistently much less than those for assimilation of ammonium, urea or free nitrogen. If nitrate-nitrogen is not so readily used as ammonium-nitrogen, the behavior of NH_4NO_3 is somewhat anomalous. One would expect a temporal change in pH and rate of nitrogen uptake, but neither is observed.

Consideration of the foregoing observations led to the following hypothesis: Cultures of *azotobacter* grown in air on nitrogen-free medium

require a period of adaptation before they can utilize at the maximum rate nitrate and probably certain organic forms of nitrogen such as asparagine. No evidence for adaptation with ammonia or urea has been as yet uncovered. In the relatively long-time total nitrogen experiments, adaptation occurs with KNO_3 before the first harvest (12 hr.), and the net effect is a period during which uptake of nitrogen is very slow. Once this lag period is completed, the rate of assimilation is comparable to that with ammonium ion so that the decrease in total nitrogen in the cells is much greater than is the apparent k value.

For example, suppose that the k value for uptake of ammonium and for nitrate after adaptation is 0.100. After 7 hours of incubation the initial nitrogen content of the ammonium cultures (approximately 1.0 mg./100 ml.) would have doubled while little change would have occurred in the cultures supplied nitrate because of the necessity for adaptation. Even though during the remainder of the experiment the rates of uptake of ammonium and nitrate are equal, the total ammonium-nitrogen assimilated will be twice the nitrate-nitrogen. Moreover, the lag period may not even be recognized since its effect at the time of the first harvest will not greatly exceed the experimental error. Also its influence on the k value will be relatively much less than on the total nitrogen so that probably all that would be noted would be a somewhat smaller k with a larger error, i.e., poorer fit of the points to the "best" line.

Confirmation of this hypothesis has been obtained in a number of experiments both macro and micro. One of its consequences is that cultures supplied with NH_4NO_3 do not change the pH or alter their rate of nitrogen uptake with time because NH_4 serves as a source of nitrogen until adaptation to NO_3 has been completed. Experiments in which the disappearance of NH_4 and NO_3 was followed by analysis for these ions disclosed little differential utilization throughout the period of greatest assimilation. During the early period the experimental error was too large to detect differences if they existed. Experiments with isotopic NH_4NO_3 will provide more information on this point.

It follows from the experiments with NH_4NO_3 that addition of a small quantity of NH_4 ion to a culture supplied nitrate should eliminate to some extent the lag period. That this actually occurs is shown by the data of Experiment 7, Table 3. When these data are graphed, as in Fig. 5, the effect is even more evident. The initial harvest was taken at 9 hours, and a distinct lag in the uptake of nitrate nitrogen was

detected. No such lag was found in the nitrate culture when a small quantity of ammonium acetate was supplied. Following the period of lag, however, the k values for uptake of nitrogen in the two nitrate cultures with and without the added NH_4 ion, were identical, but definitely less than that for ammonium acetate.

The rate of assimilation of NH_4NO_3 (not shown in the figure) was intermediate between that for ammonium acetate and potassium nitrate.

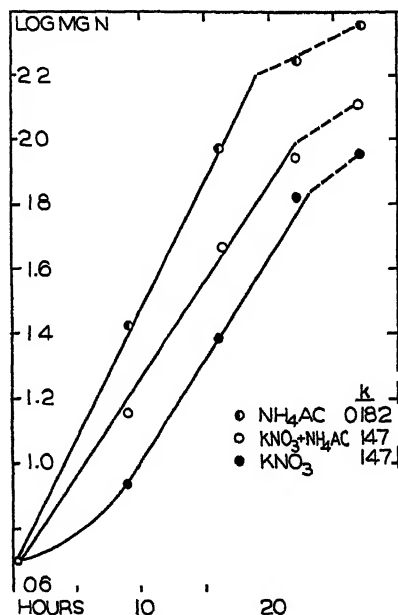


FIG. 5. Effect of presence of ammonium ion on uptake of $\text{KNO}_3\text{-N}$ by *Azotobacter vinelandii*. Note that the lag in KNO_3 assimilation is eliminated in the presence of 5 p.p.m. ammonium acetate.

But as has been mentioned before the accompanying pH changes may affect the results to some extent so that the observed differences may not be significant. Of interest is the magnitude of the k values, the highest we have ever obtained in this type of experiment.

An experiment was made to determine if the response to CO was affected when cultures were used which had been adapted to the source of nitrogen by previous transfer in its presence. The results, given in Fig. 6, are in good agreement with those already noted for the unadapted

cultures, namely, that CO does not inhibit uptake of nitrogen from potassium nitrate, asparagine or glutamate. Insufficient aspartate was available to test the effect of CO, but its utilization by cultures adapted to asparagine was determined. The k values for the various sources of nitrogen were: nitrate, 0.113; asparagine, 0.124; aspartate, 0.060; and glutamate, 0.032. The noteworthy features are the absence of a lag period in uptake of KNO_3 , although the initial harvest was taken at 7 hours in an effort to detect it, and the relatively high k value for assimila-

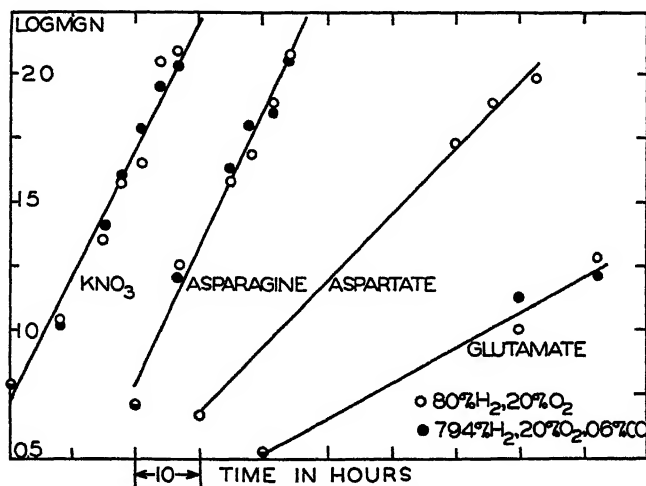


FIG. 6. Assimilation of different types of nitrogen compounds by adapted cultures of *Azotobacter vinelandii*. The cultures were adapted by transferring 5 times in a medium containing the same source of nitrogen as used in the experiment. (Asparagine culture used for aspartic acid.) Adaptation made in air, nitrogen assimilation experiment in H_2 - O_2 mixture.

tion of asparagine. Its value, of the same order of magnitude as those for NH_4NO_3 and free N_2 , is much higher than obtained with an unadapted culture. The values for nitrate, aspartate and glutamate, however, are no higher than those observed with cultures previously transferred entirely on free N_2 .

Our interpretation of these results is that adaptation occurs so rapidly with KNO_3 that its effect is not readily evident in the k value, but with asparagine a longer period is required so that previous adaptation causes a marked increase in the observed k . With aspartate and glutamate

either these are very poor sources of nitrogen for *azotobacter* or no adaptation occurred when the organism was grown in their presence in air. An effort was made to adapt to these sources of nitrogen in a H_2-O_2 atmosphere, but growth was slow and the cultures died after two to three transfers.

DISCUSSION

The principal conclusion from the results is that carbon monoxide specifically inhibits nitrogen fixation by *Azotobacter vinelandii* as had been previously established for the symbiotic red clover system. As little as 0.2% CO in the atmosphere markedly decreases fixation of N_2 , and 0.5–0.6% CO inhibits fixation 65–75% as judged by total nitrogen fixed and about 50% when based on the specific rate constant. In the same range uptake of either inorganic or organic nitrogen is not consistently affected. At higher concentrations of CO, and occasionally at 0.5%, some evidence of inhibition with combined nitrogen obtains, but it rarely exceeds the experimental error. In microrespiration studies we have found that even relatively high concentrations of CO (1–3%) do not materially increase the small inhibition observed with combined nitrogen. The pCO necessary to inhibit nitrogen fixation by *azotobacter* is about 10 times that effective with the symbiotic system, but otherwise the responses of the two types of biological nitrogen fixation parallel.

For detailed investigation of the inhibition, studies with *azotobacter* offer many advantages among which is the opportunity to test organic sources of nitrogen. In the long-time plant studies (60–90 days) we are practically restricted to ammonium and nitrate because of the difficulty of maintaining bacteriological control of the plant culture. Even with *azotobacter*, the choice is limited since it grows well only on free nitrogen, ammonium, nitrate, nitrite (low concentrations), urea and asparagine. Assimilation of the nitrogen in aspartate and glutamate is very slow, and the other common amino acids are even less available (1). In the assimilation of urea and probably asparagine, the initial step is likely an extracellular hydrolysis so that these compounds may be regarded as primarily additional forms of ammonia. Nitrate (and nitrite) likewise may be eventually assimilated as ammonia, but a reduction is involved. In view of this preference for ammonia as the source of combined nitrogen, it appears that the specificity of a given treatment for the fixation process could be established if a differential effect is observed when NH_4 compounds and N_2 serve as the nitrogen sources.

Inclusion of nitrate in the investigation provides additional information, namely, the effect of the treatment on reduction processes (in which category certain stages of N_2 fixation falls) as opposed to assimilation of reduced compounds.

In addition to these major conclusions two byproducts of the research are of general biological interest. First, certain of the results illustrate the desirability and even necessity of forming conclusions only after the rate as well as the extent of a biochemical reaction has been measured. In an early paper Werkman (4) provided an excellent example from a study of the effect of vitamin "B" on the growth of *Azotobacter chroococcum* and *Rhizobium leguminosarum*. In the studies on the mechanism of biological nitrogen fixation Burk and ourselves have repeatedly insisted that this be a necessary criterion for valid conclusions, but the significance of this criterion apparently has escaped many. Considering only the total nitrogen assimilated, we should have concluded that nitrate-nitrogen is utilized by azotobacter much less readily than either NH_4 or N_2 . The rate experiments demonstrated, however, that the apparently poor uptake of nitrate arises from an initial lag period and that once assimilation begins, its rate is probably equal to that for N_2 and not much less than for NH_4 . The occurrence and significance of the lag period would have been completely overlooked.

A second byproduct is evidence for the importance of using different techniques for mutual check in biological experimentation. In spite of the acknowledged advantages of the microrespiration method for study of biochemical reactions, the significance of the adaptation by azotobacter to source of nitrogen would have probably gone unrecognized had we not used at the same time the relatively long-time macro total nitrogen method in which adaptation had the opportunity to occur. Likewise, certain aspects of the CO inhibition which were undetected in the macroexperiments have been uncovered in the more sensitive microrespiration studies. In addition, the latter has provided us with information about certain substances which could not be tested in the macroexperiments since the quantity required was practical for use on a micro but not on a macro scale.

SUMMARY

In macro total nitrogen experiments CO inhibition of nitrogen fixation by *Azotobacter vinelandii* has been established. Definite inhibition is observed when the CO concentration is 0.1-0.2%; it increases with the

pCO until at 0.5–0.6% fixation is almost completely suppressed. This inhibition is comparable with that of inoculated red clover plants, but the quantity of CO required is about 10 times greater than for the symbiotic system.

In the range of CO concentration inhibitory for nitrogen fixation by azotobacter little consistent effect is noted on the assimilation of combined nitrogen, thereby establishing the specificity of the inhibition for nitrogen fixation. This is true for urea, ammonium and nitrate even if the experiments are made in air. With asparagine, aspartate and glutamate, however, it is necessary to use a hydrogen-oxygen mixture since fixation will predominate if N_2 is available.

Evidence is presented that azotobacter cultures kept on nitrogen-free medium in air require a period of adaptation before they readily use nitrate (or nitrite) and probably asparagine. With the adapted cultures the rate of assimilation of nitrogen sources appears to be: highest, urea and ammonium; somewhat less, N_2 , nitrate, nitrite and asparagine; much lower, aspartate and glutamate.

Certain of the findings were made possible only because rate as well as extent of fixation was measured and because two supplementary techniques—a micro and a macro—were used. The advantage and necessity of such type of experimental variation are discussed.

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Environmental Temperatures and B-Vitamin Requirements

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Last year there were reported from this laboratory (1) findings indicating a higher thiamine requirement for rats kept at 91°F. than at 65°F. Similar studies have now been carried out with others of the B fractions and the results point to a heightened need also for choline and pyridoxine under conditions of difficulty in body heat dissipation.

Choline

Male white rats—3 weeks of age—were placed in 2 well ventilated, air conditioned chambers, one of which was kept at 68°F. and the other at 91°F. and 70% relative humidity. Weighings of the individual rats and their food consumption were made twice a week. Basal diet used had the following composition:

Sucrose.....	74 g.
Casein, vitamin-free.....	18 "
Corn oil.....	2 "
Salts*.....	4 "
Thiamine.....	2 mg./kg.
Riboflavin.....	3 " / "
Pyridoxine.....	3 " / "
Pantothenic acid.....	5 " / "
Nicotinic acid.....	25 " / "
Inosite.....	1 g./ "
Para-aminobenzoic acid.....	0.3 " / "
Biotin.....	75 mcg./kg.
Haliver oil.....	1.2 cc./ "

* Salt mixture used:

Calcium carbonate.....	600 g.
Potassium phosphate, dibasic.....	645 "
Calcium phosphate, dibasic.....	124 "
Magnesium sulphate + 7H ₂ O.....	204 "
Sodium chloride.....	385 "
Ferric citrate.....	55 "
Potassium iodide.....	1.8 "
Manganese sulphate + 4H ₂ O.....	10 "
Zinc chloride.....	0.5 "
Copper sulphate + 5H ₂ O.....	0.8 "

The rats in each room were divided into groups of 4 and given access to unlimited quantities of this basal diet to which was added the follow-

TABLE 1
Effect of Choline on Food Consumption and Growth Rate

Weeks on diets	No choline		0.37 g./kg.		0.75 g./kg.		1.5 g./kg.		3.0 g./kg.		5 g./kg.	
	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain
Cold room												
First.....	48	8	64	24	66	24	79	30	80	29	62	28
Second.....	65	29	92	29	100	27	98	29	101	27	94	27
Third.....	76	27	107	36	127	47	117	36	124	39	91	26
Fourth.....	133	34	105	31	130	37	132	32	120	30	136	36
Fifth.....	115	29	114	30	113	34	142	43	114	40	111	28
Total for last 3 weeks.....	324	90	326	97	370	118	391	111	358	109	338	90
Body wt. at end of 5½ weeks.....		192		206		226		228		222		195
R Food eaten.....	3.6		3.4		3.1		3.5		3.3		3.8	
Wt. gain.....												
Hot room												
First.....	44	12	49	18	54	16	47	20	47	21	45	17
Second.....	40	7	46	14	62	22	52	21	65	27	51	22
Third.....	56	22	60	21	61	17	52	17	68	24	64	27
Fourth.....	77	42	54	13	59	22	53	21	70	25	101	34
Fifth.....	61	13	52	17	57	18	64	22	62	22	82	25
Total for last 3 weeks.....	194	77	166	51	177	57	169	60	200	71	257	86
Body wt. at end of 5½ weeks.....		133		138		149		156		174		184
R Food eaten.....	2.5		3.3		3.1		2.8		2.8		2.9	
Wt. gain.....												

ing increasing amounts of choline: group 1, no choline; group 2, 0.37 g./kg.; group 3, 0.75 g./kg.; group 4, 1.5 g./kg.; group 5, 3 g./kg.; and

group 6, 5 g./kg. Table 1 presents the group differences in growth rate and food consumption during the 5 weeks of observation.

From the data here presented it is evident that rats kept at 68°F. do best at 0.75 g. of choline per kilogram of the food mixture used; their responses at choline concentrations of 1.5 mg./kg. and 3 g./kg. are almost as good, but they do somewhat less well at 0.37 g. or 5 g. per kilogram. In tropical moist heat, on the other hand, food consumption and growth both improve steadily with rising choline concentrations in the diet. On the choline-free diet, 5 out of 8 rats died on the 9th or 10th day in the cold room and 4 out of 8 in the heat; growth of the surviving members of these control groups was very good after the danger period for acute hemorrhagic nephritis had been passed.

In order to see whether the heightened choline requirement in the heat was due to increased choline-esterase in the blood, determinations were made in 2 series of rats with the following results:

	Cc. 0.01 <i>N</i> NaOH required for 0.4 cc. serum	
	Cold room	Hot room
Series 1.....	70 (9)*	94 (8)
Series 2.....	74 (7)	98 (7)

* Figures in parentheses represent number of different blood samples used for analysis.

While choline-esterase does seem somewhat higher in the blood of hot room rats, the difference is by no means great enough to account for the marked heightening of dietary choline requirement in the hot room.

Pantothenic Acid

In studying pantothenic acid requirements at 68°F. and 91°F., the same technic and basal diets were used as with the choline series of rats with the following exceptions:

- (a) Thiamine—1 mg./kg. in the cold and 2 mg./kg. in the heat.
- (b) Pyridoxine—3 mg./kg. in the cold and 5 mg./kg. in the heat.
- (c) Choline—1.5 g./kg. in the cold and 5 g./kg. in the heat.
- (d) Pantothenic acid was omitted.

Eight groups of 4 rats each were given increasing concentrations of pantothenic acid in the diet, with the food consumption and growth findings as set forth in Table 2.

In both heat and cold there was found to be a sharp maximum in food consumption and growth at 6 mg. of the vitamin per kilogram of diet mixture through the last 3 of the 5 weeks. Both food consumption and growth showed progressive improvement up to this concentration of pantothenic acid and then declined with greater amounts. On the

TABLE 2
Effect of Biotin on Food Consumption and Growth in Rats

Weeks on diets	No biotin		37 mcg./kg.		75 mcg./kg.		150 mg./kg.		300 mcg./kg.	
	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain
Cold room										
First.....	67	29	74	30	67	28	64	28	62	26
Second..	97	32	113	37	93	33	97	34	96	30
Third.....	118	39	137	42	127	42	126	42	117	34
Fourth.....	111	33	132	33	128	31	127	36	118	28
Total for last 2 weeks.....	229	72	269	75	255	73	253	78	235	62
Body wt. at end of 4 weeks.....		178		191		178		182		161
Hot room										
First.....	42	20	43	19	46	19	62	16	38	19
Second.....	50	21	54	26	56	23	56	23	47	20
Third.....	58	22	61	25	60	23	64	20	63	24
Fourth.....	85	29	93	34	86	29	94	31	89	31
Total for last 2 weeks.....	143	51	154	59	148	52	158	51	152	55
Body wt. at end of 4 weeks.....		117		127		119		117		117

* mcg. = micrograms.

pantothenic acid-free basal diet, 3 out of 8 rats died in the hot room and none in the cold. With the 10 mg./kg. mixture there were two deaths in the hot room, and at 20 mg./kg. one death; no deaths occurred in the cold room groups.

In both heat and cold there is thus seen to be a sharp optimum in pantothenic acid concentration at 6 mg./kg. of food, with very definite toxicity at 10 mg./kg.

TABLE 3

Effect of Pantothenic Acid on Food Consumption and Growth in Rats

Weeks on diets	No panto- thenic acid		1 mg./kg.		2 mg./kg.		4 mg./kg.		6 mg./kg.		8 mg./kg.		10 mg./kg.		20 mg./kg.	
	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain	Food eaten	Wt. gain
Cold room																
First.....	84	23	60	26	66	27	67	26	92	33	65	26	86	29	86	30
Second.....	64	6	69	12	88	15	83	15	105*	35*	102	33	100	31	99	30
Third.....	55	2	55	5	80	13	95	18	101*	29*	89	22	81	17	81	21
Fourth.....	56	-2	64	3	66	9	80	14	122*	35*	78	16	81	5	94	17
Fifth.....	41	-7	61	17	76	18	75	24	136*	34*	94	34	45	-16	76	-7
Total for 3-4-5 weeks	152	-7	180	25	222	40	250	56	359*	98*	261	72	207	6	251	31
Body wt. at end of 5½ weeks		71		115		134		155		212*		189		121		140
R Food eaten																
Wt. gain.....			7.2		5.6		4.5		3.7		3.6					
Hot room																
First.....	58	18	45	21	46	22	44	21	60	21	46	22	58	17	64	18
Second.....	58	20	62	20	59	21	62	23	60	25	64	23	56	21	58	21
Third.....	49	8	58	9	66	11	69	15	72*	23*	63	15	49	14	58	21
Fourth.....	39	2	56	17	66	16	58	24	76*	25*	61	23	64	21	59	14
Fifth.....	39	2	49	10	55	16	60	22	78*	29*	64	25	70	26	65	24
Total for 3-4-5 weeks.....	127	12	163	36	187	43	196	61	226*	77*	188	63	183	61	182	50
Body wt. at end of 5½ weeks.....		97		123		136		155		168*		161		145		147
R Food eaten																
Wt. gain.....	10.6		4.5		4.3		3.2		2.9		3.0		3.0		3.1	

* Optimal food consumption and growth rates.

Biotin

The basal diet here was the same as that used in the choline study, except that the biotin was omitted and 1.5 g. of choline were added per kilogram of diet mixture used in both rooms. To different portions of the basal ration biotin was added to give 37, 75, 150, and 300 micrograms/kg. Table 3 shows the results obtained.

Growth was slightly better in both heat and cold at the lowest biotin concentration (group 2), and in two different series of cold room rats poorest growth occurred with the diet richest in biotin. The differences were slight, however, and probably of no significance. Since this biotin study was made before it was known that choline requirements are higher in the heat, all hot room groups showed deficient growth,—1.5 g. of choline per kilogram of diet having been used in both rooms.

Pyridoxine

The rat studies on pyridoxine are not yet completed, but indications are that hot room requirements are just about double those in the cold. Chicks were also used as experimental subjects in this case,—day-old white leghorn cockerels. Those selected for the cold room were kept at 78–80°F. for the first 10 days on the diets and then at 70°F. for the remainder of the observation period. Their basal diet had the following composition:

Dextrin.....	57 g.
Casein, vitamin-free.....	18 "
Salts*.....	6 "
Soy bean oil.....	5 "
Gelatin.....	10 "
Extracted liver residue (Wilson's).....	4 "
Solubilized liver residue (Wilson's).....	2 "
Cystine.....	0.2 g.
Thiamine.....	3 mg./kg.
Riboflavin.....	4 " / "
Pantothenic acid.....	15 " / "
Nicotinic acid.....	100 " / "
Choline.....	1.5 g./ "
Haliver oil.....	1.2 cc./ "

* Salts used here were composed of 4 parts rat salt mixture and 1 part dibasic calcium phosphate.

To separate portions of this basal ration were added increasing amounts of pyridoxine, with the results on growth as depicted in Table 4.

At 70°F. chick growth is practically as good at 1 mg. pyridoxine per kilogram of diet as at any higher concentration, but at 91°F. optimal growth is not reached below 4 mg./kg. Out of 3 different series of chicks run (5 to each group), 14 in the cold room on the pyridoxine-free diet died within the first 2 weeks while at 91°F. there were 10 deaths—mainly during the third week.

There was a troublesome occurrence of polyneuritis in the hot room at pyridoxine concentrations below 4 mg./kg., but none was noted among the cold room chicks. A similar appearance of polyneuritis was observed among the hot room chicks on diets with graded choline deficiency. Suspecting a thiamine inadequacy in the heat at 3 mg. of thiamine per kilogram of basal ration, we increased this to 6 mg./kg. but without

TABLE 4
Pyridoxine and Chick Growth

	Body weight in grams after 4 weeks on mg./kg. diets				
	No B ₆	1 mg./kg.	2 mg./kg.	4 mg./kg.	8 mg./kg.
Cold room.....	All dead	230 (4)*	240 (5)	224 (5)	232 (5)
Number with polyneuritis. . .	0	0	0	0	0
Hot room.	73 (3)	180 (5)	167 (2)	201 (5)	194 (5)
Number with polyneuritis....	0	2	4	0	0

* Figures in parentheses represent number of surviving chicks.

relief. Studies soon to be reported in detail have shown that 12 mg./kg. is necessary to give optimal growth and complete freedom from polyneuritis in the chicks kept at 91°F., as contrasted to the 3 mg./kg. optimum at 70°F. The nervous manifestations in the hot room chicks were worst at thiamine levels only moderately below the optimum (4 to 9 mg./kg.); none occurred in the chicks receiving only 1 mg./kg. In the chick studies on both choline and pyridoxine, some polyneuritis occurred at concentrations above as well as below the optimum for these vitamins. This phase of the study will be dealt with more fully in a subsequent article.

Riboflavin

First results with this B fraction have shown best rat growth in both heat and cold at 6 mg./kg. of food. More complete details will be

reported as soon as the series now under observation have been concluded.

DISCUSSION

Requirements of thiamine and choline for optimal growth are higher in the heat, no matter whether the calculation be based upon vitamin concentration in the diet, actual daily intake, or intake per 100 g. of body weight (see Table 5). Paired feeding experiments would have given useful information, but with these temperature differences the

TABLE 5
Optimal Vitamin Intake in Heat and Cold.

	Cold					Heat				
	Food eaten	Wt. gain	Ratio	Actual vitamin intake		Food eaten	Wt. gain	Ratio	Actual vitamin intake	
				Rat./day	100 g. body wt./day				Rat./day	100 g. body wt./day
Thiamine, 3rd, 4th weeks Cold: 0.6 mg./kg. Heat: 1.2 mg./kg.	258	54	4.8	11 mcg.	0.2 mcg.	189	55	3.1	14.4 mcg.	9.5 mcg.
Pantothenic acid, 3rd, 4th 5th weeks 6 mg./kg. in both heat and cold	359	99	3.7	103 mcg.	49 mcg.	220	77	2.9	65 mcg.	39 mcg.
Choline, 3rd, 4th, 5th weeks Cold: 0.75 g./kg. Heat: 5 g./kg.	370	115	3.1	13.1 mg.	5.8 mg.	257	50	2.9	61.2 mg.	33.3 mg.

cold room animals starved to death in about a week when fed only the amount of food consumed by those in the heat.

Growth in the heat was accomplished with less wastage of calories for maintenance of body temperature, and was most efficient in both heat and cold at the optimal vitamin concentration. It is of interest that chicks, with their higher body temperature, exhibit the same further heightening of thiamin requirement described earlier (1) for rats in fever-producing heat.

SUMMARY

Rat requirements for optimal growth are twice as high at 91°F. as at 68°F. for thiamine and pyridoxine and over 5 times as high for choline.

Chicks with their higher body temperature require 4 times more thiamine and pyridoxine for optimal growth at 91°F. than at 70°F.

With pantothenic acid, optimal rat growth was obtained in both heat and cold at 6 mg./kg. and definite toxicity at 10 mg./kg.

Biotin seems not significantly related to rat growth in either heat or cold.

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Note: The B vitamins used in this study were very kindly supplied by Merck & Co., Inc., the Haliver oil by the Abbott Laboratories, and the liver fractions by the Wilson Laboratories.

Viscosimetric Studies on the Tobacco Mosaic Virus Protein. II

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It has been demonstrated that the tobacco mosaic virus nucleoprotein shows marked anomalies in viscosity (1, 2, 3) as well as in the diffusion process (4, 2, 5, 6) in sedimentation in the ultracentrifuge (7, 8), and in solubility behavior (9, 10). Sols made up of the virus nucleoprotein are thixotropic (1, 5, 9). A picture of the mechanism of flow of this thixotropic material through the capillary of a capillary viscometer is presented in this communication, together with additional evidence bearing on the thixotropic character of the virus nucleoprotein sols.

PREPARATION OF MATERIAL

The nucleoprotein used in these studies was prepared from infected burley tobacco plants by its precipitation from the expressed sap by means of ammonium sulfate. An improved method for the isolation of the virus nucleoprotein consists in cytolyzing the plant with ether, expressing and discarding the vacuolar sap, which contains little or no virus, and then grinding the pressed plant material in a food chopper, followed by extraction of the ground material with phosphate buffer, and subsequent precipitation of the virus nucleoprotein from the phosphate buffer extract by ammonium sulfate in the usual manner. The advantage consists mainly in the initial elimination of much colored material.

The nucleoprotein was dialyzed against distilled water, and was then finally electrodialed. As the electrolytes decrease in concentration in the course of the electro dialysis, there occurs a phenomenon which is associated with the orientation of the elongated electrokinetically anisotropic protein aggregates in the electric field, namely, the precipitation of the nucleoprotein. On dispersal of the material by means of stirring, the nucleoprotein will remain in the dispersed state indefinitely if the dialysis cell be disconnected from the power line. The

nucleoprotein will reprecipitate rapidly, however, if the electro dialysis is resumed. In the final stages in the preparation of the nucleoprotein used in these determinations, the supernatant liquid in the dialysis cell was devoid of virus, had a pH of 5.2, and a specific conductance of approximately 10^{-6} . The preparation obtained after dispersal of the precipitated nucleoprotein contained 7.4 mg. of virus per ml., a pH of 4.6, and a specific conductance of 2.69×10^{-5} at 25°C .

The myosin was prepared according to the method of Gorter and Ormond (10), and the viscosity determinations were made on solutions containing 4.95 mg. protein/cc.

METHODS

The viscosity determinations were made in the manner indicated in a previous communication (1). In the case of the determinations in capillarity, the procedure consisted in cathetometrically observing the position of the meniscus in a capillary tube, on the one hand when the sol was permitted to rise spontaneously in the manner that is used in determining surface tension by the capillary rise method; and on the other, the position to which the meniscus settled when it was drawn up the tube for some distance above the rest point. The temperature was controlled thermostatically in these experiments so that the fluctuation was not greater than 0.02° . Viscosity determinations were carried out at 30° , and the determinations in the case of capillarity at 25° .

EXPERIMENTAL RESULTS

The addition of KCl to the electro dialyzed preparation had a marked effect on the apparent relative viscosity, as well as on the degree of deviation from the ideal viscosity behavior. The electro dialyzed nucleoprotein sols showed typical anomalous viscosity behavior (Curve A, Fig. 1), but in the case where sufficient KCl was added to give a 0.001 *M* solution, the deviation from the ideal viscosity behavior was particularly marked (Curve B, Fig. 1). The addition of sufficient KCl to give a 0.01 *M* solution induced a change in the apparent relative viscosity from 3.02 in case of the electro dialyzed virus nucleoprotein under a pressure gradient of about 200 dynes per cc. to something in the neighborhood of 10^4 in the presence of the salt at about the same shearing stress (Curve C, Fig. 1). Curve D is the one obtained using myosin, which, of course shows a definite anomalous viscosity as has been shown

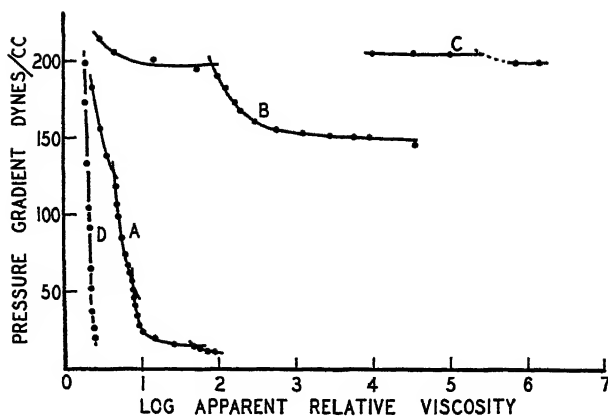


FIG. 1. Effect of KCl on the apparent relative viscosity. Curve A is for electrodialyzed virus nucleoprotein, B is for virus nucleoprotein in 0.001 *M* KCl, and curve C is for virus nucleoprotein in 0.01 *M* KCl. D is for Myosin.

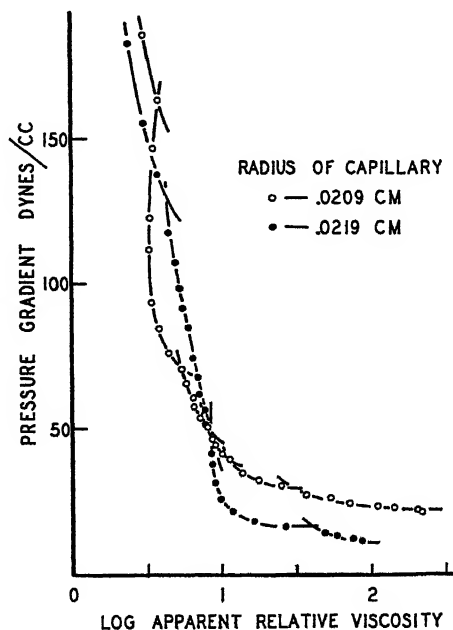


FIG. 2. Curves obtained using two different viscometers

by von Muralt and Edsall (11), and as may be seen from the curve. The data for the myosin are presented for the sake of comparison.

An interesting feature associated with these data is the indication of a discontinuous flow in the capillary of the capillary viscometer, which may be seen in both Figs. 1 and 2. In both these figures, the common logarithm of the apparent relative viscosity is plotted against the pressure gradient along the capillary of the viscometer. A flow behavior comparable to the one suggested by these data might have been anticipated in the case of thixotropic materials in particular in cases where the sol-gel transformation is as rapid as it is in the case of the tobacco mosaic

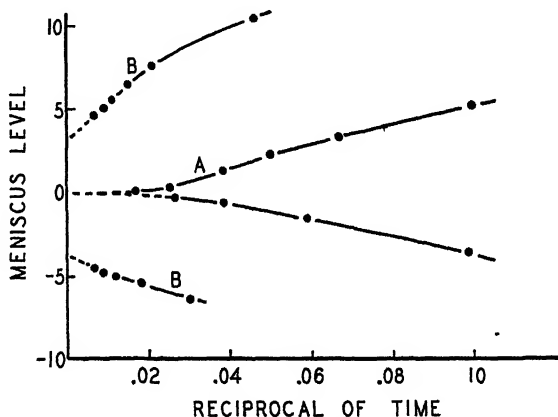


FIG. 3. Rate of rise or settlement of menisci in a capillary tube, A in the case of water, and B in the case of 1.3 per cent virus nucleoprotein at pH 5.8.

virus nucleoprotein. Bingham (12) has shown that the velocity profile of materials of this type flowing through a capillary tube is that of a truncated parabola, with the apparent streaming near the wall of the capillary where normally the velocity gradient is greatest. Unquestionably in the case of thixotropic materials flowing through a capillary tube strains are set up in the regions corresponding to the periphery of the truncated portion of the velocity parabola, and finally when rupture occurs there is a surge in the flow. The gel structure is subsequently reformed spontaneously and the process is repeated, giving one the successive series of curves in Figs. 1 and 2.

Data obtained by using two different viscometers in the case of the same electrolyzed preparation are presented in Fig. 2 for comparison.

With respect to the observations on capillarity, the method of ascertaining the rest point of the meniscus is indicated in Fig. 3, curve A, where the distance of the meniscus from the final rest point in the case of water is plotted against the reciprocal of the time; in this instance the time was in minutes. Curve B was obtained by using a preparation of the virus containing 13 mg./ml. in Luffer at pH 5.8. The radius of the capillary used was 0.01 cm.

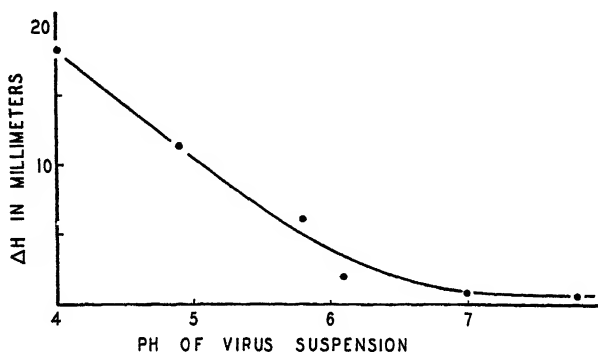


FIG. 4. Effect of pH on the relative strength of the virus nucleoprotein thixotropic gel.

The variation of the relative strength of the thixotropic gel with pH is indicated in Fig. 4. The distance between rest points of the menisci is plotted as ΔH along the ordinate.

DISCUSSION

Any adequate concept of the physical status of the various sols of the virus nucleoprotein must effect a reconciliation of the observations that have been made, and must account for all anomalies displayed. The concept of a molecularly dispersed nucleoprotein in aqueous solution which is normal in its behavior, as has been maintained by Stanley and his co-workers,¹ obviously is not adequate. The evidence from studies on biological activity, studies involving the ultracentrifuge, studies on viscosity, on diffusion, on solubility, on ultrafiltration, and finally from

¹ In justice to Lauffer, it should be pointed out that he has abandoned the concept of molecular dispersity in the case of this virus protein (13, 14), and apparently no longer has faith in the molecular weights that have been obtained from consideration of the ultracentrifugal and viscosity data.

the studies wherein the electron microscope was employed, is that the nucleoprotein as normally obtained and studied is aggregated, that the degree of aggregation is dependent on the history of the preparation, and that the aggregation is reversible to a small degree in dilute solutions. The anomalous viscosity, the anomalous diffusion, the anomalous sedimentation in the ultracentrifuge each contributes to an invalidation of the calculations of "molecular weight" from the data obtained through the use of these respective techniques. Thus the error has been made by the group at the Rockefeller Institute for Medical Research at Princeton, N. J., of attempting to use the data obtained by using the standard Ostwald viscometer in the calculation of the asymmetry constant to be used in conjunction with the sedimentation data in the calculation of the molecular weight of this protein. The value of approximately 50 million was obtained from these calculations. The fact that the value for the viscosity will depend on the manner in which it is measured has not been considered by these workers.

These various anomalies which are shown by solutions of this nucleoprotein are associated with both the thixotropic nature of the sols of the virus nucleoprotein and the aggregation, and the thixotropy has tended to mask the effects of aggregation so that an uncritical consideration of the data, in particular those relating to the use of the ultracentrifuge, has led to the concept of a molecularly dispersed macroprotein molecule. Some of the evidence bearing on the general problem may be summed up briefly.

If the leaves of some varieties of plants are inoculated with the virus by rubbing the leaf surfaces gently with a cloth that has been moistened with the virus preparation, local necrotic lesions will appear on the inoculated leaves. As a rule preparations containing about 10^{-7} g. of the nucleoprotein per ml. are on the threshold of infectivity, but with some preparations the threshold occurs with 10^{-6} g. per ml., even though other leaves of the same plant, or even other portions of the same leaf may show infection with a second preparation containing much less nucleoprotein. Preparations containing as little as 10^{-14} g. per ml. have proved infective, but this high infectivity cannot be reproduced at will (15, 16). The curve obtained by plotting the number of lesions per unit of area of leaf surface against the dilution of a sample of the virus follows the equation

$$y = N(1 - e^{m\Delta})$$

where y is the number of lesions obtained at a given dilution m , and N and A are constants. The equation was derived independently by both Youden (17) and Bald (18) from statistical considerations, and apparently is free from objections. The assumptions involved in its derivation seem reasonable. In the above equation N represents the maximum number of lesions the leaf will show when the surface is saturated with the virus. Normally one would expect a variation in N since the physiology of the leaf is involved. A , on the other hand, is a factor which is related to the number of infective units per unit volume of solution, and in experiments standardized with respect to quantities of nucleoprotein used, the value of A should be constant if the infectivity is a constant property of the nucleoprotein and the extent of aggregation may be neglected. Values of A , obtained from the various local lesions-dilution data in the literature that were obtained by using the isolated nucleoprotein, vary irrationally between 4,000 and 320,000 when the data are expressed in terms of grams of virus nucleoprotein per cc. of inocula. One may suspect that at least a part of the variation in A is associated with the variation in the degree of aggregation of the nucleoprotein, but, parenthetically, it does not seem likely that so large a variation may be attributable completely to the variation in the degree of aggregation.

In the face of the implications of the infectivity data, significance has been attached to the apparent homogeneity that has been observed in studies involving the ultracentrifuge. The conclusion that certain of the preparations were homogeneous with respect to size was reached from mere qualitative inspection of the boundary (7), and calculations of molecular weight have been made accordingly. It has not been emphasized, however, that samples containing 0.05 per cent nucleoprotein or less contain material that does not sediment in the ultracentrifuge in particular if the preparations are permitted to stand, whereas the same preparations in more concentrated solutions show homogeneity with no nonsedimentable material (7). Obviously in the more dilute solutions the aggregation is reversed. This particular observation relative to the nonsedimentable material is interesting, in view of the statements by Stanley (19, 20) that anomalies in viscosity and diffusion have been demonstrated in relatively concentrated solutions and that it is unreasonable to extrapolate the anomalies to the more dilute solutions that have been used in the ultracentrifuge studies. Anomalies in viscosity and diffusion have been observed in preparations

of the tobacco mosaic virus nucleoprotein containing 0.05 per cent (1) and 0.02 per cent (4) respectively!

It seems probable that the apparent homogeneity observed in the ultracentrifuge is to be attributed to the thixotropy of the preparations; indeed as the preparations are made more and more concentrated the rigidity of the thixotropic gel increases and the rate of sedimentation in a given field decreases. Lauffer has attempted to extrapolate the curves, obtained by plotting the sedimentation constant against the concentration, to infinite dilution (8) in an effort to ascertain the sedimentation constant for the dynamically independent particles. One would judge from the graph presented in his paper that Lauffer made no attempt to determine the sedimentation rate of the "nonsedimentable" material that appears in preparations containing 0.05 per cent or less virus nucleoprotein. One may well question the justification for the extrapolation he proposes. His data do account, however, for the rather wide variability in the values for the sedimentation constant for this virus that have been reported, since the rate of sedimentation is a function of the concentration.

In line with the apparent reversal of the aggregation of the nucleoprotein suggested by the ultracentrifuge studies, data obtained from diffusion studies carried out by using the refractometric method of Lamm (21) support the suggestion that the aggregation is reversible in the more dilute preparations. One gets the feeling from the skewed diffusion curves that on the solvent side of the original boundary in regions where there is a high dilution that the diffusion material experiences an acceleration in the diffusion process. One may suspect that the phenomenon may be accounted for in part on the assumption that some of the aggregates have broken up to smaller units that diffuse more rapidly.

Of interest in this connection are the data on the streaming double refraction shown by sols of the virus nucleoprotein. The streaming double refraction of the virus nucleoprotein varies from preparation to preparation (22, 23, 24), and apparently a given preparation may be fractionated into components giving different intensities of streaming double refraction. In their paper on streaming double refraction, Lauffer and Stanley (24), give a value of 23.0 for the streaming double refraction of a preparation that had been precipitated by means of the ultracentrifuge four times; the supernatant liquid above the fourth precipitate contained virus nucleoprotein that was given a value of 14.3 for its streaming double refraction.

With respect to the ultrafiltration data, a consideration of the fraction of the total virus that filters through a given ultrafilter can hardly be considered seriously as criteria either of the size of the ultimate virus particle or of the state of aggregation of the nucleoprotein, but rather it is the *end point* of filtration that may be of value in determining the approximate size of the active agent. The argument advanced by Stanley and his coworkers, relative to the soundness of the concept of molecular dispersity of this nucleoprotein, which is based on data obtained from the use of only a single ultrafilter (22) does not carry weight. Indeed the various carefully conducted ultrafiltration experiments, in particular those of Thornberry (25), strongly support the suggestion recently advanced (26) that the smaller units appearing in all the electron microscope photographs of this virus protein, namely those having dimensions 15×37 millimicrons, represent the approximate maximum size that may be assigned to the infectious unit. Electron microscope photographs of the biologically active ultrafiltrates obtained near the filtration end points probably would throw some light on the problem.

SUMMARY

1. The degree of anomaly in the viscosity of the tobacco mosaic virus nucleoprotein is greatly increased in the presence of dilute KCl.
2. The flow of the sol of the virus nucleoprotein through a capillary is discontinuous.
3. The degree of thixotropity is increased markedly with decreasing pH.

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Some Sulfanilamide Antagonists as Growth Factors for Lactic Acid Bacteria

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Woods (1) first postulated that p-aminobenzoic acid was an essential metabolite in living organisms because this substance was highly effective in preventing the antibacterial action of sulfanilamide. Extracts of natural origin had previously been known to exert a similar action to greater or less degree (2). That this action of natural extracts was at least partially due to presence of p-aminobenzoic acid was shown by the isolation of this substance from yeast extract by Blanchard (3). Meanwhile, Rubbo and Gillespie (4) discovered that p-aminobenzoic acid was necessary for the growth of *Clostridium acetobutylicum*, thus greatly strengthening the supposition that this substance was an essential metabolite for living organisms in general.

That substances other than p-aminobenzoic acid are present in natural extracts which antagonize sulfanilamide action is, however, well recognized (5, 6). Harris and Kohn (6) have shown that methionine exerts such an action, while very recently Martin and Fischer (7) have indicated that adenine or hypoxanthine is as active (on the weight basis) in antagonizing the protective action of sulfanilamide against infections in mice as is p-aminobenzoic acid.

In a previous paper (8), it was demonstrated that certain of the purine and pyrimidine bases exhibited growth-factor activity for several lactic acid bacteria, including *Lactobacillus arabinosus*. Isbell (9) has recently shown that under certain conditions p-aminobenzoic acid stimulates acid production (and presumably growth) by this organism. An investigation of the interrelationships of these compounds in their growth-promoting action for certain lactic acid bacteria and in their antagonistic action toward sulfanilamide inhibition was therefore undertaken, with results indicated below.

EXPERIMENTAL

Test Organisms and Media. The cultures used were *Lactobacillus arabinosus* 17-5; *Lactobacillus pentosus* 124-2, *Lactobacillus casei*, *Streptococcus lactis* R and *Leuconostoc mesenteroides* P-60. *L. casei* was grown at 37°, the other organisms were grown at 30°. Inocula and conditions of testing were those described by Snell, Guirard and Williams (10). For growth tests, the base medium previously described (10) was modified by omitting the purine bases (adenine sulfate and guanine hydrochloride) and adding 3.0 mg. of pyridoxine hydrochloride to Solution 3. The hydrolyzed casein was treated twice with activated charcoal as described (10) to insure the absence of traces of adsorbable vitamins (11) and of p-aminobenzoic acid (9). Biotin was added as S.M.A. concentrate 1000; the folic acid preparation used was a concentrate of potency 2000 (12). This constitutes "Medium A," which was used for most of the experiments. For special purposes, a second medium was used (Medium B) which contained crystalline biotin in place of biotin concentrate, and a folic acid preparation of potency 13,000.

Growth Promoting Action for Lactic Acid Bacteria of Substances Antagonistic to Sulfanilamide. As mentioned above, p-aminobenzoic acid, certain purine bases, and dl-methionine have been shown to act antagonistically to sulfanilamide. Results tabulated in Table I show that for certain lactic acid bacteria (*L. arabinosus*, *L. pentosus*) each of these compounds is individually capable of directly promoting growth. The great dilutions at which p-aminobenzoic acid is active justify its classification as a true growth-factor for these organisms (9), even though their requirement for it is quite non-specific. The metabolic interrelationship between p-aminobenzoic acid and methionine previously postulated (6) appears sound, and could be extended to include each of the purine bases studied here. At higher levels methionine inhibits growth completely. The growth-promoting action of methionine or of the purine bases for these organisms cannot be attributed to p-aminobenzoic acid contained as an impurity, since solution, charcoal treatment and recrystallization did not change their activity. Hypoxanthine was prepared from adenine by treatment with nitrous acid (13) which would destroy any p-aminobenzoic acid present as an impurity.

Other organisms of the lactic acid group (*S. lactis*, *L. mesenteroides*) are specific in their requirement for the purine bases; p-aminobenzoic acid or methionine has no effect on their growth under these conditions. It is interesting that *L. mesenteroides* cannot utilize adenine for growth

TABLE I

The Effect of p-Aminobenzoic Acid, Methionine and Purine Bases on Growth of Various Lactic Acid Bacteria

Substance added	Amount	Galvanometer reading*				
		<i>L. arabinosus</i>	<i>L. pentosus</i>	<i>S. lactis</i>	<i>L. mesenteroides</i>	<i>L. casei</i> †
	<i>γ per 10 cc.</i>					
	0	8.8	19	10	4.0	74
	0	9.6	19	10	4.0	74
p-Aminobenzoic acid	0.001	33	58	10	4.0	74
	0.01	71	73		4.0	74
	0.10	71	70	11	4.0	
	1.00	71	70		4.0	
	10.00	71	72	10	4.0	74
dl-Methionine	10	12	21	10	4.0	74
	30	25	26		4.0	
	100	59	62	10	4.0	74
	300	75	74		4.0	
	1,000	8.0		10		
Adenine sulfate	10	58	77	28	4.5	74
	30		80		4.0	
	100	69	82	29	4.5	71
Guanine hydrochloride	10	67	74	20	30	74
	30		77		53	
	100	76	82	29	75	71
Xanthine	10	67	67	17	39	74
	30		77		53	
	100	72	77	25	72	70
Hypoxanthine	10	72	73	18	32	74
	30		79		50	
	100	79	82	43	65	68

* This is a quantitative measure of culture turbidity. Zero reading is 100% transmission; a reading of 100 represents complete opacity. All cultures were incubated 24 hours. Medium A was used.

† 0.1% asparagine was added to medium A for this organism (14, 15).

purposes, whereas guanine, xanthine and hypoxanthine are highly effective. The presence of these compounds is of no value for *L. casei*, under the conditions here used.

Because of the apparent non-specificity of the growth response of *L. arabinosus*, a number of other compounds were tested for ability to promote growth of this organism under these conditions. These are listed

TABLE II

*The Effect of Purine Bases, Methionine, and p-Aminobenzoic Acid on Reversal of Sulfanilamide Toxicity for Lactobacillus arabinosus**

Sulfanilamide	Reversing agent (Growth factor)	Reversing agent				Galvanometer reading			
		(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
γ per 10 cc.		γ per 10 cc.	γ per 10 cc.	γ per 10 cc.	γ per 10 cc.				
0	p-Aminobenzoic acid	0	0.001	0.003	0.01	9.0	33	65	71
20	p-Aminobenzoic acid	0	0.3	1.0	3.0	1.0	1.5	26	68
0	Adenine sulfate	0	1.0	10	100	9.0	45	58	69
20	Adenine sulfate	10	100	1000	30,000	1.0	1.0	1.0	1.0
20	p-Aminobenzoic acid	0.3	0	0.3	0.3	1.0	1.0	39	69
	plus Adenine sulfate	0	10	1.0	10				
0	Hypoxanthine	0	1.0	10	100	9.0	45	72	78
20	p-Aminobenzoic acid	0	0.3	0.3	0.3	1.0	62	73	79
	plus Hypoxanthine	100	1.0	10	100				
0	Xanthine	0	1.0	10	100	9.0	23	67	72
20	p-Aminobenzoic acid	0	0.3	0.3	0.3	1.0	19	60	75
	plus Xanthine	100	1.0	10	100				
0	dl-Methionine	0	30	100	300	9.0	9.0	24	61
20	p-Aminobenzoic acid	0.3	0.3	0.3	0.3	1.0	1.0	1.0	1.0
	plus dl-Methionine	30	100	300	1,000				

* Incubation time—24 hours. Medium A was used.

below together with the highest level (per 10 cc. of medium) at which they were tested: asparagine 30 mg.; o-aminobenzoic acid 10 γ ; m-aminobenzoic acid 10 γ ; thymine 300 γ ; p-aminophenylacetic acid 30 γ ; urethane 10 mg.; pimelic acid 100 γ ; inositol 100 γ ; and choline chloride 100 γ . All were inactive.

Antisulfanilamide Action of Purine Bases as Determined with Lactic Acid Bacteria. Because of the equivalence of the purine bases, p-aminobenzoic acid and dl-methionine in producing growth of *L. arabinosus* and of *L. pentosus*, and the reported efficacy of these same compounds in antagonizing sulfanilamide action for various other organisms, it seemed of interest to examine the action of these compounds in reversing sulfanilamide toxicity for various lactic acid bacteria. Results secured with *L. arabinosus* are given in Table II. In the presence of 20 γ per culture of sulfanilamide, approximately 1000 times as much p-aminobenzoic acid is required to produce growth as in the absence of sulfanilamide. Although in the absence of sulfanilamide, the purine bases and methionine exert the same growth-promoting action as does p-aminobenzoic acid, no such action is detectable in the presence of sulfanilamide, even when these compounds are tested at levels 1000 times as high as those promoting growth on the sulfanilamide-free medium. At such high levels of concentration, these compounds may, of course, show toxicity, and thus mask any antagonistic action which they might otherwise show. When the purine bases were tested for their effect in the presence of amounts of p-aminobenzoic acid just insufficient to show reversal of sulfanilamide toxicity, each of the bases tested was effective in the reversal of sulfanilamide bacteriostasis. This reversal must be ascribed to a specific antagonism of these bases to sulfanilamide. Thus in growth effect (in the absence of sulfanilamide) 100 γ of adenine sulfate is approximately equivalent to 0.01 γ of p-aminobenzoic acid; whereas in antagonistic action to sulfanilamide (in the presence of suboptimal amounts of p-aminobenzoic acid) 10 γ of adenine is equivalent to more than 2 γ of p-aminobenzoic acid. Guanine, although not included in the table, showed the same reversal action as did adenine, hypoxanthine and xanthine. Methionine showed no reversing action even in the presence of suboptimal amounts of p-aminobenzoic acid. Urethane was similarly tested, but was without effect.

In further tests on the efficacy of purines in reversing sulfanilamide toxicity, *Lactobacillus pentosus* was used (Table III). With this organism and medium A (biotin concentrate), adenine alone was effective in reversing sulfanilamide toxicity, the effective concentration being from 100–300 times that producing a corresponding level of growth in the absence of sulfanilamide. With p-aminobenzoic acid, this ratio is approximately the same. Hypoxanthine, xanthine and guanine were as effective as adenine. The effect was, however, somewhat erratic:

occasionally the purines failed to show definite reversal of the sulfanilamide effect. In an attempt to locate the cause of this variability, the experiments were repeated with medium B (crystalline biotin). Growth

TABLE III
*The Effect of Adenine and p-Aminobenzoic Acid on Reversal of Sulfanilamide Toxicity for Lactobacillus pentosus**

Sulfanilamide γ per 10 cc.	Reversing agent	Reversing agent γ per 10 cc.	Galvanometer reading	
			Medium A	Medium B
0	p-Aminobenzoic acid	0	9.0	7.2
		0.001	34.0	30.0
		0.010	55.0	49.0
		0.1	60.0	51.0
20	p-Aminobenzoic acid	0	4.0	1.1
		0.1	18.0	4.4
		0.3	58.0	17.0
		1.0	61.0	48.0
		3.0	62.0	52.0
0	Adenine sulfate	0.1	25	23
		0.3	42	38
		1.0	56	55
		3.0	64	57
20	Adenine sulfate	3		2.0
		10	17	2.2
		30	41	2.2
		100	47	2.2
		300	50	2.2
20	Adenine sulfate†	0		4.4
		0.3		13.5
		1.0		32.0
		3.0		50.0

* Incubation period: 24 hours.

† In presence of 0.1 γ p-aminobenzoic acid per tube.

effects of the purine bases and of p-aminobenzoic acid occurred at the same concentrations as previously, as did the reversal of sulfanilamide toxicity by p-aminobenzoic acid. On this medium, however, adenine

alone was completely ineffective in reversing sulfanilamide toxicity. The same was true of hypoxanthine, xanthine and guanine. In the presence of 0.1 γ of p-aminobenzoic acid per tube (an amount insufficient by itself to show reversal of the sulfanilamide effect) adenine was highly effective in reversing the sulfanilamide toxicity. Under these conditions, 3 γ of adenine had as great an effect as 1 γ of p-aminobenzoic acid.

These results clearly indicate that the biotin concentrate used in medium A contains a substance which permits the purine bases to exert sulfanilamide antagonism. The biotin concentrate in Medium A supplies 8 γ of solids per tube, the nature of which is unknown. The effect

TABLE IV

*The Effect of Purine Bases and p-Aminobenzoic Acid on Reversal of Sulfanilamide Toxicity for Lactobacillus casei**

Sulfanilamide	Reversing agent	Reversing agent				Galvanometer reading			
		(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
		γ per 10 cc.	γ per 10 cc.	γ per 10 cc.	γ per 10 cc.				
γ per 10 cc.									
0						74	74		
10,000						8.0	8.0		
10,000	p-Aminobenzoic acid	0.1	0.3	1.0	3.0	30	43	53	44
10,000	Adenine sulfate	0.1	1.0	10	100	16	25	45	45
10,000	Guanine hydrochloride		1.0	10	100		41	57	47
10,000	Hypoxanthine		1.0	10	100		46	63	50
10,000	Xanthine		1.0	10	100		54	55	51

* Medium A plus 0.1% asparagine. Incubation period: 36 hours.

is probably not due to p-aminobenzoic acid, for the presence of 0.001 γ of this substance produces marked effects on the growth of both *L. arabinosus* and *L. pentosus* on either medium used in this investigation, while the substitution of biotin concentrate for pure biotin in medium B results in no growth increase with either organism.

Separate experiments with *L. pentosus*, which it seems unnecessary to report in detail, showed the same effect on reversal of sulfa-drug toxicity by the purine bases whether sulfanilamide, sulfathiazole or sulfapyridine was employed as the bacteriostatic agent.

Lactobacillus casei provides an interesting case for testing the effect of purines and p-aminobenzoic acid on reversal of sulfanilamide bacteriostasis, since under the conditions used, these compounds had no effect on growth (Table I). In preliminary tests it was found that this

organism was highly resistant to sulfanilamide bacteriostasis; the presence of 10,000 γ per tube was required to effect complete bacteriostasis during a 24 hr. growth period. This compares with 20 γ per tube required to produce the same effect with *L. arabinosus* and *L. pentosus*. The effect of p-aminobenzoic acid and of the purine bases on sulfanilamide bacteriostasis of this organism is presented in Table IV. Under the conditions used, each of the purines tested reverses sulfanilamide bacteriostasis to the same degree as does p-aminobenzoic acid, which they approach closely in activity. Separate tests showed that urethane had no effect in concentrations up to 1000 γ per 10 cc. while dl-methionine was fully effective at this level.

DISCUSSION

The results secured above support fully the findings of Martin and Fischer (7), who found that adenine and hypoxanthine were antagonistic to the action of sulfanilamide in mice. Their results indicated that guanine was inactive in this respect; with the lactic acid bacteria here used, guanine and xanthine are both effective.

On purified media, the antisulfonamide action of these substances is shown most clearly in the presence of amounts of p-aminobenzoic acid insufficient by themselves to show reversal of sulfanilamide bacteriostasis. Clearly, this is a situation which would exist in the animal body if, as has been postulated (1), p-aminobenzoic acid is present in animal tissues. It is also probable from the difference in results secured with Medium A and B that natural materials contain substances other than p-aminobenzoic acid in the presence of which reversal of sulfanilamide toxicity by purine bases occurs.

The fact that each of the naturally occurring compounds so far found to antagonize sulfanilamide action can replace each other in their direct effect on growth of certain lactic acid bacteria is added evidence for some metabolic interconnections between these substances.

SUMMARY

Conditions are detailed under which a direct growth-promoting action of p-aminobenzoic acid, dl-methionine, adenine, guanine, xanthine and hypoxanthine can be observed with *L. arabinosus* and *L. pentosus*. For these organisms, any one of these compounds is sufficient to permit growth. Other members of this group (*S. lactis*, *L. mesenteroides*) appear specific in their requirement for one or more of the purine bases.

In the presence of suboptimal quantities of p-aminobenzoic acid, the purines listed above are effective in reversing sulfonamide bacteriostasis of *L. arabinosus* and *L. pentosus*. With *L. pentosus* and *L. casei*, reversal of sulfonamide bacteriostasis is effected by the purine bases in the absence of added p-aminobenzoic acid. For *L. pentosus*, evidence is presented which indicates that this action of purines is dependent upon a naturally occurring substance of unknown nature, which is probably not p-aminobenzoic acid.

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The Activation of Papain¹

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Mendel and Blood (6) established the activation of papain by hydrogen cyanide and by hydrogen sulfide and demonstrated that this action was reversible. Many experimenters have since confirmed these observations and extended the list of activators and of inhibitors. The problem of the mechanism originated when Willstätter and Grassmann (10) compared the action of hydrogen cyanide on papain to that of enterokinase on trypsinogen and postulated a new enzyme of extended activity. Krebs (5) believed activation was due to the removal of traces of heavy metals which would otherwise act as inhibitors; however, Murray (8) demonstrated that the effects of copper upon a variety of samples of papain were identical although hydrogen cyanide activated the same samples to various extents. Mothes (7), Hellermann and Perkins (4), Purr (9), and Bersin (2) postulated that activation resulted from the reduction of the disulfide linkage in the inactive papain molecule to the thiol grouping of the active form. Balls and Lineweaver (1) report that cystine and the iodoacetate ion inactivate crystalline papain and explain this as the destruction of the free thiol group in the active enzyme. Fruton and Bergmann (3) have recently criticized the theory of thiol activity.

In the following a quantitative study of the relation of activator concentration to activity is presented, which furnishes additional data on the activation of papain.

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EXPERIMENTAL

Determination of Proteolytic Activity. The substrate was a purified gelatin from the Eastman laboratories labeled "Compressed Foam," containing 0.02% ash, a 1% solution in water having a pH of 4.9. The final reaction mixture consisted of 4% gelatin, 0.016% papain, 0.55 *M* acetate buffer (pH 4.71) and 0.0122 *M* HCN. After about two hours, the amino nitrogen set free in 1 cc. of the mixture was determined by a 20 minute reaction with nitrous acid in the micro Van Slyke apparatus, and the volume of gas so produced was reduced to *S.T.P.*

The time necessary to liberate this volume of gas was compared with the time necessary for a standard sample to produce the same volume, and the inverse ratio of the times observed, in per cent, indicated the relative activity of the unknown sample. The standard sample was an unactivated purified papain of high activity.

When activators other than 0.0122 *M* HCN were used, correction was made for the amount of nitrogen gas liberated in each case. A correction was also made for stability of the enzyme where required. Activation prior to hydrolysis was found to be unnecessary. Contrary to observations of Willstätter and Grassmann (10), prior activation had no effect as shown in the following table:

Prior activation time	Relative activity
<i>hours</i>	
0.03	200
0.52	213
1.00	201
1.50	186
2.92	212
3.45	221
4.02	180

Tests of the Method of Determination. The above method of determining papain activity was tested in two ways. In one case the amount of enzyme present was altered and accordingly the relative activity was found to vary linearly with the concentration (Fig. 1). In the second case a series of hydrolysis curves with various activators is drawn, and the time of the determinations on these curves multiplied by the relative activity of the enzyme so activated. The points so obtained lay along the hydrolysis curve of the standard sample (Fig. 2).

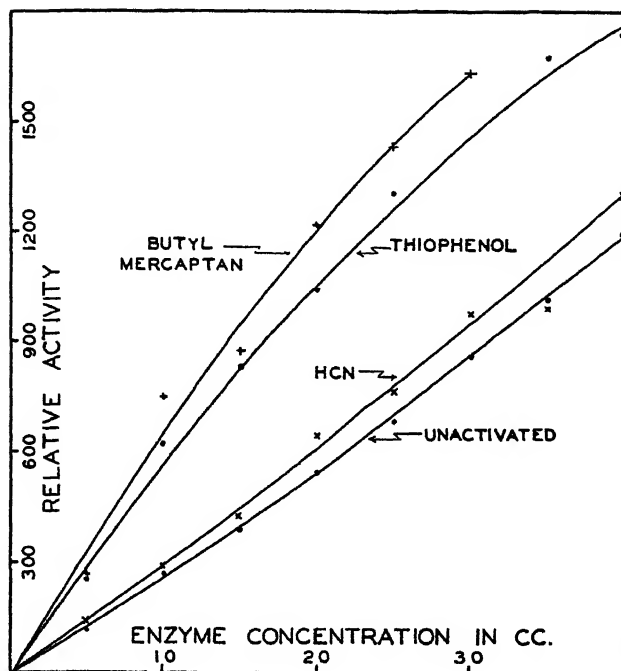


FIG. 1. Relation between enzyme concentration and activity

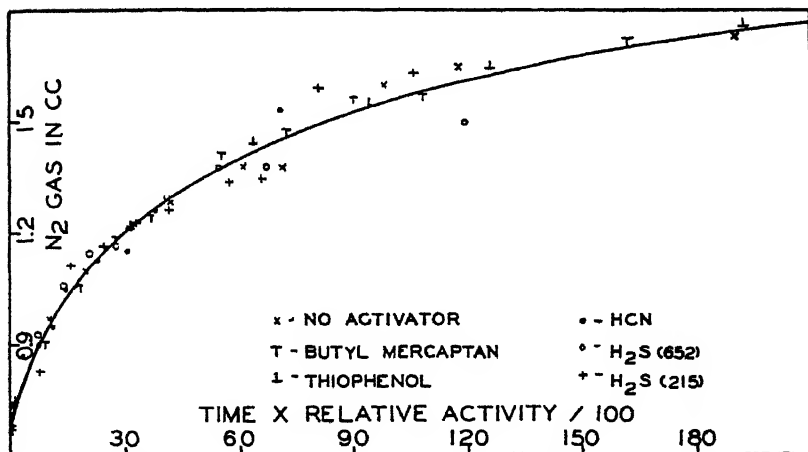


FIG. 2. Validity of the second method of determining papain activity

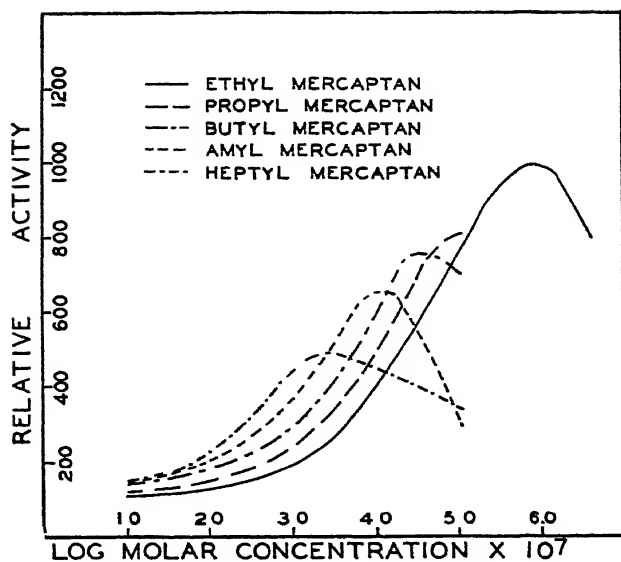


FIG. 3. Activation by mercaptans

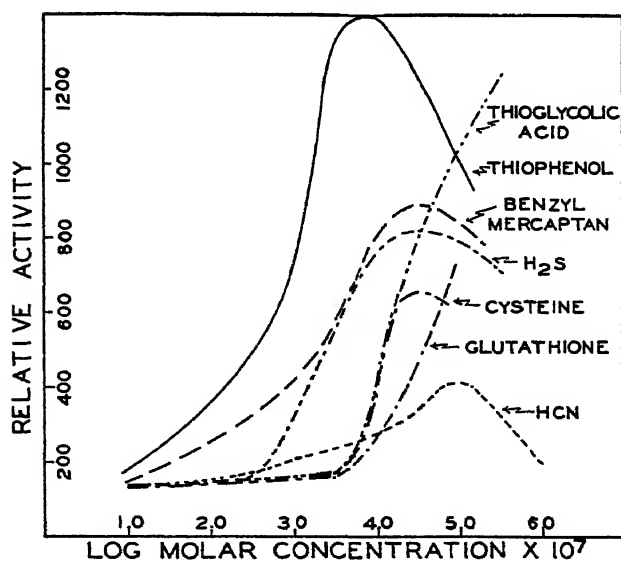


FIG. 4. Activation by thiols and HCN

These results show that both the hydrolysis curve and the method of determining activity are independent of the mode of activation.

Preparation of Papain. After trials with numerous variations the following procedure was adopted. Commercial papain was dissolved in five times its weight of 5% NaCN brought to pH 4.8 with glacial acetic acid. When papaya latex was used, 25 g. NaCN were added to 300 cc. of the latex; this solution was brought to pH 4.8 with glacial acetic acid and made up to 500 cc. The mixture was allowed to stand overnight and filtered. To the solution were added 35 g. ammonium sulfate

TABLE 1
Combinations of Activators

(Activators used: 5.80×10^{-4} M butyl mercaptan; 5.00×10^{-5} M heptyl mercaptan; 1.58×10^{-3} M HCN; 1.46×10^{-4} M H₂S; 6.60×10^{-5} M thiophenol)

Activator	Relative activity	
	Observed	Calculated as additive
Butyl mercaptan.....	388	
Heptyl mercaptan.....	293	
HCN	300	
H ₂ S	451	
Thiophenol.....	403	
Butyl plus heptyl mercaptans.....	1,200	557
Butyl mercaptan plus HCN.....	313	564
Butyl mercaptan plus H ₂ S	576	715
Butyl mercaptan plus thiophenol	1,132	667
Thiophenol plus H ₂ S.....	456	730
Thiophenol plus HCN.....	310	579
HCN plus H ₂ S.....	247	627

for each 100 cc. solution, and the resulting precipitate removed. This precipitate was dissolved in a small amount of water and dialyzed in a sausage casing for one day against running tap water and for one day against running distilled water. The solution was then filtered and to it was added five times its volume of acetone. The resulting precipitate was again filtered off, dried and ground.

Five commercial samples of papain tested had relative activities of 131, 126, 100, 99, and 63, respectively; two commercial samples purified by this method, 240 and 206, and a sample of papaya latex similarly purified had an activity of 236.

Experiments on Activation. The enzyme preparation used was commercial, purified as above, and with a relative activity of 240. The activators were added in a series of 16 to 20 concentrations of which each concentration was one-half the preceding one. The results are presented in Figs. 3 and 4. The curves of the aliphatic mercaptans were verified by a repetition of the experiment.

Certain activators were added together to the enzyme and their combined effect on the enzyme noted (Table I).

It was attempted to determine the platinum electrode potential at which activation took place. However, it was found that neither solutions of enzyme plus activator nor of activator partly oxidized by iodine solution would poise a platinum electrode.

DISCUSSION

It has been shown above that the activation of papain is not a time reaction. Since this is true, if activation is an oxidation-reduction reaction, it must either be a reversible or a stoichiometric one. A stoichiometric process is improbable because at least a 10,000 fold molar excess of ethyl mercaptan over papain is required to fully activate the latter.

From the evidence in this paper it is possible to indicate three mechanisms by which papain may be activated.

(1) The papain molecule may be reversibly reduced by its activators to give a much more effective catalyst than was the original molecule. This reversible oxidation-reduction is unusual in that it takes place at no definitely-poised potential; furthermore, at high concentrations, the activators inhibit papain to various extents.

(2) The papain molecule may combine with the activator to form a complex of unknown nature which is a catalyst of enhanced activity. The increase in efficiency may be a function of the nature of the activator.

(3) The activator may, by its presence at the interface of the papain molecule, increase the proportion of peptide bonds split when these latter approach the interface. The activators have a tendency to concentrate in the interface, and when this concentration is high enough, they block the approach of substrate molecules and thus inhibit the hydrolysis.

The third explanation seems preferable both because it is more comprehensive than the others and because there is in Fig. 3 an indication that a surface effect is present. For it is there evident that the homologous mercaptans activate papain in the same order of concentration as an homologous series of heteropolar compounds lowers the surface tension of water. Only by this third mechanism can one explain why the higher homologs activate both at a lower concentration and to a lesser extent than do the lower homologs.

SUMMARY

1. A study of the relation of concentration of a group of papain activators to the amount of activation produced has been made.

2. From the data thus assembled, it has been concluded that the activation of papain may be a surface phenomenon.

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Interference Between Bacterial Viruses

I. Interference between Two Bacterial Viruses Acting upon the Same Host, and the Mechanism of Virus Growth*†

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INTRODUCTION

The growth of a bacterial virus (Bacteriophage), occurring only in the bacterial cell, may be said to proceed behind a closed door. The experimenter can follow the virus up to the moment it enters the cell, and again after liberation from the cell. There is, as yet, no way of telling what goes on within the cell, except by circumstantial evidence which covers the entry of the virus into the host, its time of stay, its exit, and, perhaps, the metabolism of the host cell.

By the desire to gain more direct insight into the intracellular processes of virus growth, the present authors were led to try the simultaneous action of two different viruses upon the same host cell. There was a possibility that one virus might lyse the cell, while the other was still growing. Thus, an intermediate state of virus growth would be revealed. This expectation did not materialize. Instead, a striking case of interference was discovered, which could be analyzed in some detail. This will be reported in the present paper.

The growth of each virus alone in the host cell was also studied. Taken together, these results permit some conclusions concerning the mechanism of virus growth.

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† The second paper of this series will be published in the next issue of this journal.

‡ Fellow of the John Simon Guggenheim Memorial Foundation.

Our attention was drawn to previously described cases of interference in animal and plant viruses (1, 2, 3, 4). We believe that the case of interference between bacterial viruses, which we describe in this paper, may help to clarify the general problem of virus interference.

EXPERIMENTAL TECHNIQUE, MATERIAL AND PROCEDURE

For the interpretation of the experimental results a detailed acquaintance with the material used, with the principles underlying the experimental procedures, and with the quantitative analysis of the results, is indispensable. Therefore, after a brief description of the routine technique, we will discuss these items in detail.

Technique

Medium for liquid cultures: Difco nutrient broth (8 g./liter) + 0.5 per cent NaCl.

Solid medium: 1 per cent powdered agar in the above broth.

Bacterial assay by colony count. 0.1 cc. of a suitable dilution is spread, or "plated," on agar in 10 cm. Petri dishes. The colonies are counted after 24 hours incubation at 36.5°C.

Virus assay by plaque count. Suitable dilutions of the unknown are mixed with a heavy suspension of bacteria from a 24-hour slant. 0.1 cc. of the mixture is spread on agar. The virus produces holes or "plaques" in the bacterial film. Each plaque is a colony of virus particles, which has grown from a single infective center. The method is the precise analogue of the bacterial colony count method. An infective center may be either a particle of virus or a bacterium infected with virus (5).

All platings for bacterial and virus assay are made in duplicate.

Adsorption measurements are made by centrifugation of 1 cc. of a diluted sample of the mixture of virus and bacteria (four minutes in a universal centrifuge, at 3600 r.p.m.) and comparison of the amount of virus originally present with that remaining in the supernatant.

All broth cultures are kept in water bath at 36.5°C. and are continuously aerated by bubbling sterile filtered air through the cultures. This also ensures uniform mixing of the culture.

Material

Two different viruses, α and γ , were used, both of which are active upon a common bacterial host, B (*E. coli*).¹

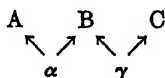
¹ These strains were kindly sent to the junior author by Dr. J. Bronfenbrenner. Originally virus α was called P28, and virus γ was called PC. Our choice of names, an adaptation to our experiments, will presently be justified. Virus γ (PC) has been purified and described by Kalmanson and Bronfenbrenner (6).

On agar, α produces large plaques (0.5–2 mm. diameter), visible after 6 hrs. incubation. After 24 hrs. incubation they are surrounded by a large halo. γ produces small plaques (0.2–0.5 mm.) visible after 24 hrs. incubation. The differences between the two viruses will be discussed in a later section. They differ markedly both in size and structure (12).

Bacterial "indicator strains" are needed in order to follow the growth of each virus in cultures in which both are present with the host. These indicator strains were obtained by the following method. It is a well known fact that the lysis of a bacterial culture is rarely complete. Usually a few hours or days after the first clearing, a secondary growth arises, which can be isolated in pure culture. This new strain is usually resistant to the action of the virus in the presence of which it arose. The sensitivity of such a variant to other viruses may be the same as that of the primary strain. Accordingly, two variants, A and C, of our bacterial host B, were obtained from secondary growths after the action of γ and α . Strain A was found to be unchanged in its sensitivity to α and completely resistant to γ , and conversely, strain C was found to be unchanged in its sensitivity to γ and completely resistant to α . Adsorption experiments showed that strain A does not adsorb γ and strain C does not adsorb α .

Sometimes, secondary cultures are lysogenic, i.e., they are carriers of the virus in the presence of which they have been isolated. A and C were tested for lysogenicity. Diluted bacterial cultures as well as their filtrates were plated with the sensitive strain B; no plaques were obtained. The possibility of their being lysogenic was thus excluded.

The relationship between the bacterial strains and the viruses may be summarized in the following scheme, in which the arrows indicate sensitivity. The symmetry of this scheme is the justification for our choice of names.



When a mixture of the two viruses is plated on agar with each one of the three bacterial strains, the plates seeded with A show only the large plaques of α , those seeded with C show only the small plaques of γ , and the plates seeded with B show both. The plates from such an experiment are shown in Fig. 1.

The system shown in the above diagram enabled us to study the growth of α and γ on the common host, both separately, and under conditions of mixed infection.

The Procedure for Virus Growth Experiments

The purpose of the growth experiments is to obtain information about a certain number of measurable quantities, which characterize the "life-cycle" of the virus in a sensitive host (7).

The first step of this life-cycle in a mixture of virus and bacteria is the adsorption of the virus on the sensitive bacterial cells. This will sometimes be referred to as the "infection" of the bacteria. The adsorption rate is measured by assay-

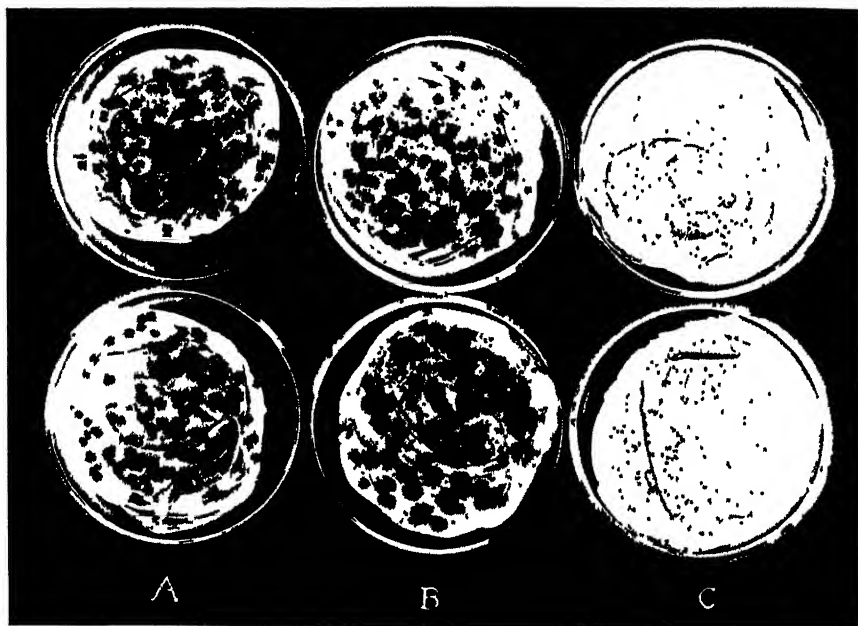


FIG. 1. Mixture of viruses α and γ plated with the three bacterial strains A, B, C. The large plaques are colonies of virus α , the small of virus γ . Strain A is the indicator strain for virus α , strain C for virus γ .

ing at suitable intervals the virus in the supernatant after centrifugation of the mixture. Under definite experimental conditions (physiological state of the bacteria, temperature, medium) the adsorption rate is proportional both to the virus and the bacterial concentration (8, 9, 10). This relationship is of fundamental importance for the design of growth experiments, since it shows that the adsorption rate can be reduced, at any desired moment, to any desired extent simply by a dilution of the reacting mixture of virus and bacteria. For instance, suppose we have, in a certain mixture, an initial adsorption of 50 per cent of the virus in five minutes. If the mixture is diluted 1:1000, the relative adsorption rate will be reduced by the same factor and further adsorption will proceed at the

negligible rate of 1 per cent in 100 minutes. By a heavy dilution at the proper moment one can, therefore, terminate the adsorption period, measure the amount of adsorption obtained, and study the destiny of the bacteria infected.

The second phase of the life-cycle is the multiplication of the virus in the cell. After the bacterial cell has adsorbed a virus particle, it retains normal appearance for a while, then, suddenly, the newly formed virus particles are liberated. In most cases the cell is lysed at the same moment. Therefore, if one follows the number of infective centers by the plaque-counting technique, one finds a period, the *constant period*, in which apparently nothing happens, and a second period, the *rise period*, in which the plaque count rises sharply. After this, the newly liberated virus particles will become adsorbed to other bacteria still present in the culture, unless this is avoided by previous high dilution of the culture, as explained above. The average number of virus particles liberated from an infected cell will be called the *burst size*.

1. The method for determining quantitatively the elements which characterize the life-cycle (adsorption, constant period, rise period, burst size) will now be described by discussing one growth experiment of the type used throughout this work.

Table I is the schedule of a growth experiment called *one-step growth* (5), because one isolates in this experiment one step in the growth of the virus, namely the step of the liberation of virus from the bacteria infected during a short initial adsorption period. The essential element of the schedule is a heavy dilution of the mixture of bacteria and virus after a few minutes of contact. By this dilution one achieves two aims, viz., one limits infection to a period which is small compared to the constant period, and one avoids the complicating effects of reinfection of the remaining bacteria by the virus liberated.

The results of the experiment given in Table I may be analyzed in the following manner.

The titers (in units per cc.) of bacteria or of virus are obtained by multiplying the colony count or the plaque count for the 0.1 cc. samples by ten times the factor of dilution.

The bacterial assay shows that the experimental bacterial culture B_{exp} contained 5×10^7 B/cc. two minutes before the virus was added. The stock virus α had a titer of 3.25×10^9 particles/cc. At the time zero, 0.2 cc. of the stock was added to 20 cc. of B_{exp} to form the adsorption mixture B_a . Therefore this mixture contained

$$\begin{aligned} &5 \times 10^7 \text{ bacteria/cc.} \\ &3.25 \times 10^7 \text{ virus particles/cc.} \end{aligned}$$

At the time five minutes, B_a was diluted 1:2000 to form the growth-tube I, and 1:200,000 to form the growth-tube II. Further adsorption in these growth-tubes is negligible. The assay of the supernatant from the centrifuge tube shows that at this time the tube B_a contained

$$1.70 \times 10^7 \text{ unadsorbed virus particles/cc.}$$

Therefore, 1.55×10^7 virus particles/cc. had been adsorbed. This is 48 per cent of the input, and gives an average of

$$0.3 \text{ virus particles adsorbed per bacterium.}$$

TABLE I

Schedule of Experiment No. 6. One-step Growth Experiment of the Virus α

Time minutes	
-150	0.05 cc. of a 24 hrs. broth culture of B is inoculated into 20 cc. broth, and incubated at 37°C. with aeration. This is the experimental culture, tube B _{exp} .
-10	Assay of the stock virus α : 0.1 cc. of dilution 1/10 ⁶ plated with bacteria washed from 24 hrs. slant. *1—337 plaques 2—316 "
-2	Assay of B _{exp} : 0.1 cc. of dilution 1/(5 × 10 ⁴) plated. *3—94 colonies 4—104 "
0	0.2 cc. of stock virus α added to B _{exp} . This is the adsorption mixture, tube B _a . The time 0, at which virus and bacteria are mixed, marks the beginning of the timing from the virus growth.
5	0.02 cc. of B _a added to 2 cc. of broth at 37°C. in a centrifuge tube. From this tube <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <p>1 cc. is added to 19 cc. of broth, and aerated at 37°C. This is the first growth-tube, I.</p> </div> <div style="width: 30%;"> <p>0.1 cc. from tube I added to 9.9 cc. of broth at 37°C. This is the second growth-tube, II.</p> </div> <div style="width: 30%;"> <p>1 cc. is centrifuged. Supernatant diluted 1/100, and 0.1 cc. plated with bacteria. *5—166 plaques 6—174 "</p> </div> </div> <p>Samples from tubes I and II taken at intervals, diluted 1/10 and 0.1 cc. plated with bacteria for virus count.</p>
8	*7—143 plaques 8—151 "
11	9—129 " 10—136 "

* Serial numbers of the Petri plates.

The operation of sampling, mixing with bacteria, and plating two plates takes about 50 seconds. The timing is so arranged that the plating of the first plate coincides with the schedule time.

TABLE I—*Continued*

Time minutes			
15	11—1500 plaques 12—1400 “		
17		*13— 47 plaques 14— 44 “	
19		15— 66 “ 16— 62 “	
22		17— 89 “ 18— 83 “	
25		19—106 “ 20— 91 “	
27		21— 89 “ 22— 94 “	
30		23—109 “ 24— 85 “	
41		25— 95 “ 26— 86 “	
50		27— 90 “ 28—114 “	

Since this is small compared to unity, only a negligible fraction of the bacteria had adsorbed more than one virus particle, and only about one-third of the bacteria were infected.

Owing to the dilution, the initial titers of bacteria and of virus in the two growth-tubes were:

	Tube I	Tube II
Bacteria/cc.....	2.5×10^4	2.5×10^2
Virus particles/cc.....	1.62×10^4	1.62×10^2

The later assays from these tubes, that is, the titers of infective centers (free virus particles + infected bacteria) after different times, are plotted in Fig. 2, in which this experiment is represented by the open circles. The plaque titer is plotted against time, relative to the initial titer. Such a plot we shall call a *One-step growth curve*. It is seen that the titer stays constant for 13 minutes. During this time the virus grows in the cell, but is not released from it. At 13 minutes the titer begins to rise and increases in ten minutes by a factor of 62.

This part of the growth curve represents the liberation of virus from the cells, which in this case are also lysed during this interval. All the cells that liberate virus in this interval were infected during the initial adsorption period. After the ten-minute rise period the titer again remains constant. This is, as ex-

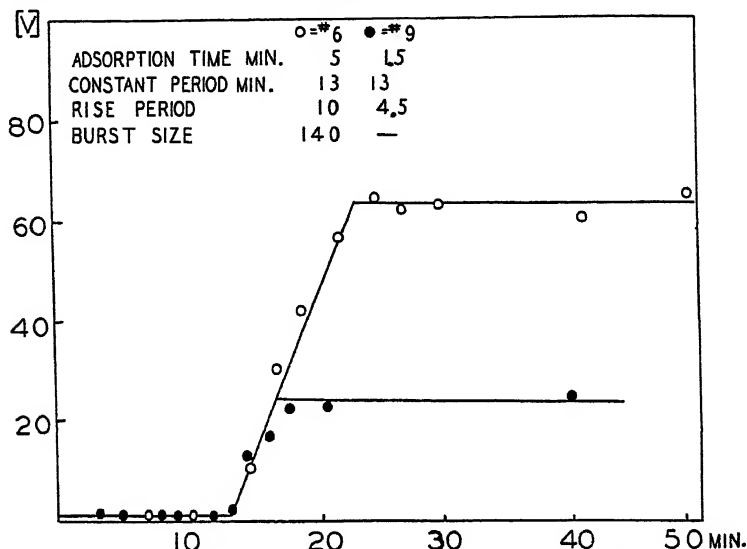


Fig. 2. One-step growth curves of virus α , single infection. \circ = experiment no. 6. \bullet = experiment no. 9. [V] = relative titer of virus.

plained above, because of the high dilution in the growth-tubes, which prevents readsorption.

We can now list the quantitative results:

Constant period..... 13 minutes

Rise period..... 10 minutes

Step size (final titer:initial titer)..... 62

The step size does not represent the yield of virus per infected bacterium. It has to be corrected for the fraction of virus which was not adsorbed during the initial adsorption period and which, therefore, had no chance to infect a bacterium and to grow. This fraction has to be subtracted, both from the initial and from the final titer.² Thus the average yield of virus per bacterium or the

$$\begin{aligned}
 (1) \quad \text{average burst size} &= \frac{\text{final virus} - \text{initially unadsorbed virus}}{\text{initial virus} - \text{initially unadsorbed virus}} \\
 &= \frac{(184 - 1.70) \times 10^7}{(3.00 - 1.70) \times 10^7} = 140.
 \end{aligned}$$

² For the initial titer the values obtained from the stock assay and from the assays during the constant period are averaged.

The above is an example of the typical experiment of virus growth. Several modifications of it have been used, which must now be described.

2. The most important modification is that of "multiple infection" in contradistinction to "single infection."

We have seen that the ratio of the number of adsorbed virus particles to the number of bacteria gives us the average number of virus particles adsorbed per bacterium (0.3 in the above example). If the bacteria are greatly in excess, practically no bacteria will be infected by more than one virus particle, and we speak of "single infection." On the other hand, we have seen that the rate of adsorption is proportional to both the concentrations of virus and of bacteria. Therefore, if we keep the concentration of bacteria constant, and increase the concentration of virus, adsorption will increase in direct proportion. A limiting factor might be the saturation of the bacterial cells with virus. Such a saturation phenomenon has been observed, but only at much greater concentrations than any used in our present experiments (11).

Under conditions of multiple infection several new quantitative elements become important. The first of these is the multiplicity of infection:

multiplicity = virus particles adsorbed/bacteria.

The second is the number of uninfected bacteria. If, on the average, n virus particles are adsorbed per bacterium, the fraction of uninfected bacteria will be, according to Poisson's law, equal to e^{-n} . For instance, if n equals four, there will be $e^{-4} = 1.8$ per cent uninfected bacteria:

uninfected bacteria per cent = $100e^{-n}$ (n = multiplicity).

A possible limitation of this calculation will be discussed later with experimental data.

In multiple infection experiments, the plaque count titer drops during the initial adsorption period, because nearly every bacterium collects several virus particles, but produces only one plaque. For this reason the burst size, i.e. the yield of virus per infected bacterium, cannot be evaluated by the method described in the case of single infection. Let us call

I = Input of virus

U = Unadsorbed virus

F = Final titer of virus

B = Bacteria initially present

B_i = Initially infected bacteria

P = Plaque titer during the constant period.

The burst size is then by definition

$$(F - U)/B_i.$$

The first four and the sixth of the above quantities are directly determined during the experiment. The fifth one has to be obtained indirectly. In the case of single infection, it is determined as

$$B_i = I - U,$$

since every adsorbed virus particle will infect a different bacterium; the burst size is then

$$(F - U)/(I - U).$$

The formula was used in the example on page 118.

In the case of multiple infection, B , will be smaller than $I - U$, since the bacteria have adsorbed more than one virus particle each. On the other hand, if the multiplicity of infection is rather high, practically all the bacteria will be infected, and B , can be replaced by $I - U$. Therefore the burst size becomes

$$(F - U)/B.$$

The value of B is usually measured a few minutes before the beginning of the experiment, and has to be corrected for the small increase between this time and the average time of adsorption. In most cases this correction, not exceeding 10-20 per cent, could be neglected, since it is of the same order as the sampling errors.

P , the plaque titer during the constant period, must be equal to the sum of the titers of infected bacteria and of unadsorbed virus:

$$P = U + B, \text{ or}$$

$$P = U + B \text{ if all bacteria are infected.}$$

Let us illustrate these considerations by an example.

Experiment 28. Virus γ on B , multiple infection.

$$I = 17.0 \times 10^8/\text{cc.}$$

$$B = 0.67 \times 10^8/\text{cc.}$$

$$U = 6.8 \times 10^8/\text{cc.}; I - U = 10.2 \times 10^8/\text{cc.}$$

$$\text{multiplicity} = 15.$$

$$B + U = 7.47 \times 10^8/\text{cc.}$$

$$P = 7.5 \times 10^8/\text{cc.}$$

$$F = 175.0 \times 10^8/\text{cc.}$$

$$\text{Burst size} = (F - U)/B = 250.$$

3. Experiments on mixed infection of bacteria with both viruses are carried out as those described above, except that samples are alternately plated with the indicator strains A and C, in order to obtain separate growth curves for α and γ . The analysis of these experiments will be considered in connection with experimental results.

4. In some experiments it was desired to follow the growth of the uninfected bacteria parallel to the growth of the virus. Samplings were made for colony count assays and plated after suitable dilution. Small amounts of virus in these samples do not usually interfere with the bacterial count. Bacteria, which at the moment of sampling are infected but not yet lysed, will not form colonies.

Miscellaneous Experiments

1. The growth of the bacterial strains A, B, and C was studied by following complete growth curves, starting with very dilute suspensions in broth of bacteria from 24 hour aerated cultures. The lag phase, the time of division during the logarithmic phase, and the saturation titer were calculated in the usual way from such growth curves.

2. Microscopic observations of the living bacteria, both infected and uninfected, were made by spreading a suitable dilution of the culture on the surface

of nutrient agar Petri dishes, covering with a coverslide, and observing with oil immersion objective. Periodical observations of the microscopic field (generally at room temperature) were recorded on hand drawn maps.

EXPERIMENTAL RESULTS

1. Growth of the Bacterial Strains A, B, and C

It was necessary, for the virus growth experiments, to work with a reproducible standard phase of the growth cycle of the bacterial strains. The bacterial growth was studied in cultures incubated with continuous aeration, and was found to be quantitatively reproducible. The results are summarized in Table II. The division time is obtained from the slope of the growth curve in the log phase, and corresponds to the time

TABLE II
Growth of the Bacterial Strains

Strain	Lag period	Division time in the logarithmic growth phase	Maximum titer in the stationary phase
	hours	minutes	B/c.
A	1.3	26	3.8×10^9
B	1.5	19	4.3×10^9
C	1.3	19	2.1×10^9

required for a twofold increase in titer. For experiments on virus growth, bacterial suspensions should be used with a titer well below the saturation value, but at the same time, as high as possible, in order to give good adsorption of virus in a few minutes. Bacterial concentrations between 3 and 10×10^7 /cc. satisfied these conditions.

It is worth noting that the division time of the indicator strain C is the same as that of the primary strain B, whereas that of the indicator strain A is considerable longer (26 minutes as compared with 19 minutes). The strain A is the one which was isolated from the secondary growth after lysis of B with virus γ .

2. Growth of the Virus α

The experiments were performed as explained in the section on procedure. Experiments with bacteria in excess (single infection), and with virus in excess (multiple infection) will be reported separately.

(a) *Single Infection.* Results are summarized in Table III. Experi-

ment 6 is the one described in detail in Table I, and in Fig. 2, which also shows Experiment 9. The adsorption in these experiments was always about 50 per cent in five minutes. In Experiment 9 the adsorption time was only 1.5 minutes. The adsorption was, therefore, small and could not be accurately determined. For the same reason, the burst size was not estimated.

It will be seen that, for growth on strain B, the constant period is accurately reproducible, with an average of 13 minutes. This value is a characteristic of the system B + α under our standard conditions. We will see later that it is not changed by multiple infection.

The rise period is fairly reproducible (7.5 to 10 minutes) in different experiments with the same adsorption time of five minutes. When only 1.5 minutes are

TABLE III
Growth of Virus α . Single Infection Experiments

Experiment	Adsorption time	Adsorbed virus	Constant period	Rise period	True rise period	Burst size
No.	minutes	per cent	minutes	minutes	minutes	
6	5	45	13	10	5	140
7	5	58	13	9	4	144
8	5	*	12.6	7.5	2.5	*
9	1.5	*	13	4.5	3	*
Average.....			13		3.6	142

* Not measured.

allowed for adsorption, the rise period is reduced to four minutes. This is proof that the length of the rise period is, in part, determined by the length of the adsorption period. Earlier infected bacteria liberate the virus earlier. The true variability of the period between adsorption and liberation of virus is given by the difference between the rise period and the adsorption period. It is, therefore, only about three minutes and a half. The value thus obtained will be called the "true rise period." The burst size, *i.e.*, the average yield of virus particles per infected bacterium, is also well reproducible, with an average of 142.

(b) *Multiple Infection.* The results are given in Table IV.

It will be seen that the adsorption rate is the same as in the experiments with single infection. This means that, even for the highest multiplicities attained in these experiments, the bacterial surface is still well below the point of virus saturation.

The constant period is precisely the same as that for single infection, 13 minutes. The rise period is shorter, if one compares experiments with equal

adsorption periods. This is to be expected because, with virus in excess, practically all the bacteria will be infected at least once within a very short time, probably within the first minute.

The burst size is larger than for single infection (203 as compared with 142), a difference well outside the limit of experimental errors. The burst size does not show any correlation with the multiplicity of infection.

The increase in burst size may have the following simple explanation. Let us suppose that the bacteria can still divide after infection almost until lysed. In multiple infection, both daughter cells of such a division will, in general, be infected, whereas in single infection only one of them may be infected. In multiple infection the actual number of infected bacteria will, therefore, be larger than the number given by the bacterial assay, and therefore the total yield of virus will be increased proportionally. Quantitatively, this explanation runs as follows: the burst size is increased by 45 per cent; this would call for an increase in the number of bacteria of 45 per cent. Such an increase would require 10

TABLE IV
Growth of Virus α . Multiple Infection Experiments

Experiment	Adsorption time	Adsorbed virus	Multiplicity of infection	Constant period	Rise period	Burst size
No	minutes	per cent		minutes	minutes	
12	5	49	6	13	7	220
13	5	45	9	13	4.5	175
14	3	50	11.5	12.5	4	215
Average	13	5	203

minutes, just three minutes less than the constant period of virus growth. We would have to assume, then, that bacteria can divide until three minutes before lysis.

(c) *Microscopic Observations.* Bacteria, multiple infected with virus α , were transferred, shortly before the end of the constant period, to agar plates and were observed under the microscope. The first cells were lysed about fifteen minutes after infection and all were lysed 28 minutes after infection. The slight delay of the onset of lysis, as compared to the onset of virus liberation (a delay of about two minutes), can probably be ascribed to the lower temperature at which the observation takes place.

3. Growth of the Virus γ

(a) *Single Infection.* Results are summarized in Table V. One experiment is shown in Fig. 3 (solid circles).

The rate of adsorption for virus γ is a little higher than for virus α , 70–80 per cent is adsorbed in five minutes.

TABLE V
Growth of Virus γ . Single Infection Experiments

Experiment	Adsorption time	Adsorbed virus	Constant period	Rise period	True rise period	Burst size
No	minutes	per cent	minutes	minutes	minutes	
19	5	80	21	8	3	140
21	5	75	21	9	4	130
18	3	45	21	9	6	135
22	1.25	18	21.5	5	3.75	*
Average		21		4.2	135

* Not measured.

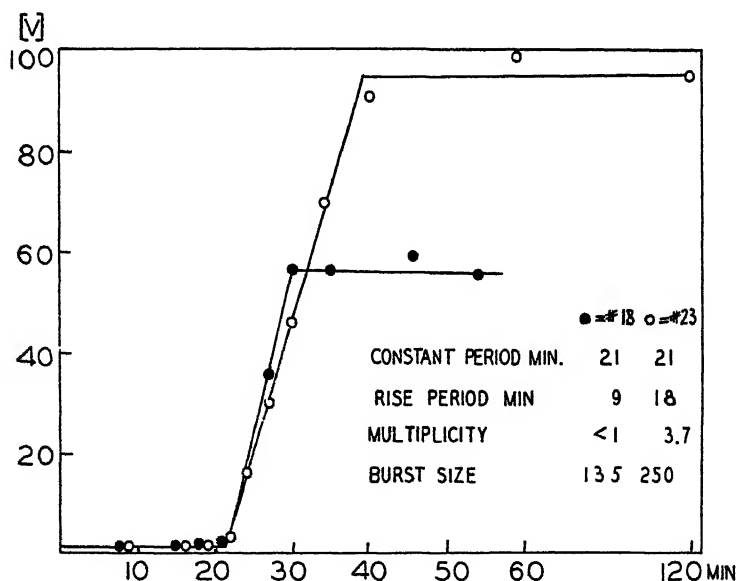


FIG. 3. One-step growth curves of virus γ . ● = experiment no. 18, single infection. ○ = experiment no. 23, multiple infection. [V] = relative titer of

The constant period is 21 minutes and is again accurately reproducible. .

The rise period depends on the adsorption time, as with virus α . The true rise period (the variability of the period between infection and virus liberation) is about four minutes.

The burst size is well reproducible, with an average of 135. The difference between this value and the one found for virus α , 142, is within the limits of experimental error. In the case of virus γ , however, the calculation of the burst size is not quite unambiguous, because the plaque titer does not always stay accurately constant after the main increase has occurred. A slow continuous rise, amounting to about 20 per cent, may follow the first steep rise. This slow rise of the titer may be due to a small amount of readorption on previously uninfected bacteria in the growth-tubes. The burst sizes given above are calculated from the titers at the end of the steep rise, disregarding the further slow increase.

(b) *Multiple Infection.* Results are given in Table VI. Experiment 23 is shown in Fig. 3 (open circles).

TABLE VI
Growth of Virus γ . Multiple Infection Experiments

Experiment	Adsorption time	Adsorbed virus	Multiplicity of infection	Constant period	Rise period	Burst size
<i>No</i>	<i>minutes</i>	<i>per cent</i>		<i>minutes</i>	<i>minutes</i>	
23	5	65	3.7	20 5	18	250
24	5	61	6.3	21	13	250
28	5	60	15	23	16	235
30	5	65	15	22	20	315
Average				21.5	16 5	262

The adsorption rates and the constant period are the same as for single infection, as in the case of virus α .

The rise period, which in the case of virus α , was, as expected, shortened by multiple infection, is in this case considerably lengthened. We are unable to give a plausible explanation of this result. We suspect that it is in some way connected with the fact that the constant period for this virus is longer than the division time of the bacteria.

The average burst size is 262, an increase of about 100 per cent over the burst size for single infection. As in the case of the other virus, the burst size shows no correlation with the multiplicity of infection. Here, too, the increase may be due to the division of multiple infected bacteria. The division of the bacteria would have to continue until a few minutes before virus liberation, in order to account for the large increase.

(c) *Microscopic observations* show a strict correlation between virus liberation and lysis, both with regard to the onset and completion of these two processes. All bacteria are lysed within the expected time limits.

4. *The Survival and Growth of the Uninfected Bacteria in the Growth Tubes*

The purpose of these experiments was twofold. First, to see whether the titer of viable bacteria, in a mixture of bacteria and virus, drops in proportion to the infection of the bacteria. Second, to see whether the uninfected bacteria continue to grow while the virus titer remains constant after virus liberation. Most of these experiments were done with virus γ , and the results of these will be given first.

(a) *Virus γ* . In two experiments of the one-step growth type with virus in excess, the bacterial titer was followed by platings parallel to the virus titer platings. The following facts were observed:

1. An initial decrease of the bacterial titer, as expected on the hypothesis that infected bacteria are eliminated by lysis.

2. A slight retardation in the division rate of the uninfected bacteria, lasting between 20 and 40 minutes. Control experiments with bacteria in the absence of virus, showed the same retardation. It is, therefore, to be ascribed to manipulation of the culture, when it is transferred, diluted, etc.

3. Normal growth of the uninfected bacteria after this period, throughout the remainder of the experiment.

It was desired to check whether the initial diminution of the bacterial titer is in quantitative agreement with the theoretical expectation. The fraction of uninfected bacteria according to Poisson's formula, should be e^{-n} , where n is the multiplicity of infection, *i.e.*, the average number of virus particles adsorbed per bacterium. The applicability of this formula depends upon two conditions. First, it must be assumed that the bacteria are all equal in their affinity toward the virus. Second, the plaque count titer must not only be proportional to the number of virus particles in the suspension, but must be actually equal to it, *i.e.*, the efficiency of plating (5) must be unity.

An experiment was performed, in which different amounts of virus were added to the same number of bacteria, the adsorption was measured, and the surviving fraction of bacteria determined by colony count.

Results of the whole group of experiments are summarized in Table VII.

It will be seen that the calculated and the experimental titers of the surviving bacteria are similar in all cases; in some cases they agree closely. The deviation between experimental and theoretical values is greatest for high multiplicity of infection, the experimental titer in

these cases being smaller than the expected values. Deviations can be accounted for by assuming that the true virus titers are not more than 20 per cent higher than the values given by plaque assays.

(b) *Virus α*. Only one growth curve was followed with bacterial and virus assays in parallel. The initial decrease of the bacterial titer was of the expected magnitude. The growth of the uninfected bacteria, after a slight retardation, continued normally as in the case of virus *γ*.

These results confirm our picture of the infection of the bacteria by the virus particles and of the elimination of infected bacteria by lysis. Moreover, they justify the use of Poisson's formula for the calculation of the fraction of uninfected bacteria. This will be used frequently in the experiments reported in the next section.

TABLE VII
Survival of Bacteria in the Presence of Virus γ

Experiment	Initial bacterial concentration	Adsorbed virus	Multiplicity of infection	Surviving bacteria	
				Experimental	Calculated
<i>No.</i>	<i>B/cr</i>	<i>V cr.</i>		<i>per cent</i>	<i>per cent</i>
25a	8.0×10^7	1.9×10^7	0.24	75	79
25b	8.8×10^7	6.0×10^7	0.68	30	49
25c	8.3×10^7	23.5×10^7	2.9	2.7	5.5
26	8.0×10^7	15.5×10^7	1.9	12.5	15
27	7.3×10^7	29.0×10^7	4	0.7	1.8

5. Mixed Infection of Bacteria with Virus α and Virus γ

A variety of experiments were made with different ratios between the bacterial and virus titers. It may be well to state at the outset that there was never found any interference between the two viruses as regards *adsorption*. The adsorption rates for both viruses α and γ were found the same, or nearly the same as in the experiments with either virus alone.

(a) *Mixed Infection with Both Viruses Greatly in Excess*. An experiment of this type is shown in Fig. 4. The bacteria were mixed at time zero with both viruses in excess, such that, at the end of the adsorption period, the multiplicity of infection was $(4.2\alpha + 4.3\gamma)/\text{bacterium}$.

It will be seen that there is hardly any increase of virus α, whereas the increase of virus γ takes place as in the absence of virus α (constant period 21 minutes, rise period 15 minutes, burst size 220). The amount of virus α found in the first samples is equal to the measured amount of *unadsorbed* virus α. Later, it is increased

only by a factor 1.5. In the absence of virus γ , there would have been an increase of the titer of virus α by a factor of 40-50 in this experiment. The small increase of virus α which does take place is completed at 17 minutes, well before the beginning of the rise of the γ -titer.

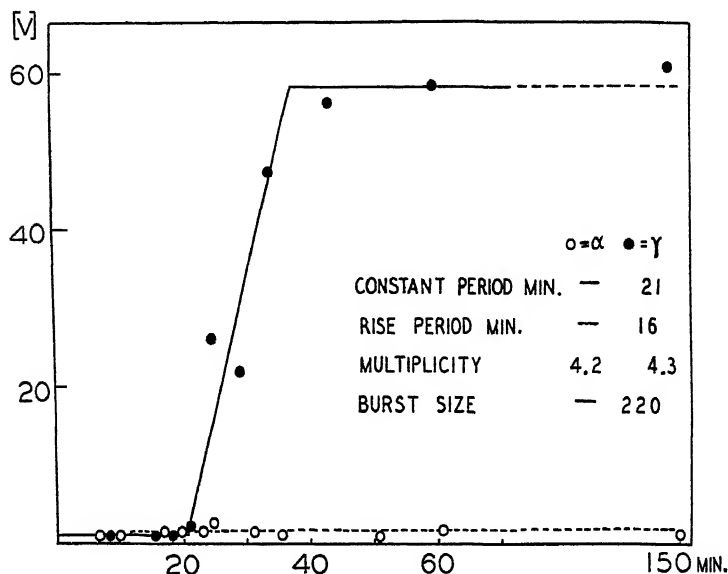


FIG. 4. Experiment no. 34. Mixed infection. Bacteria plus viruses α and γ in large excess. \circ = virus α . \bullet = virus γ . $[V]$ = relative titer of virus.

The experiment suggests that there is interference between γ and α , such that mixed infection of a bacterium by γ and α leads to the suppression of virus α .

(b) *Mixed Infection with Both Viruses Slightly in Excess.* In the experiment shown in Fig. 5, the mixture of bacteria and viruses was set up in such proportions, that at the end of the adsorption period the multiplicity of infection was only $(1.55\alpha + 1.16\gamma)$ /bacterium. Under these conditions, a sizeable fraction of the bacteria will not be infected with virus γ , namely $e^{-1.16} = 31$ per cent, using Poisson's formula.

Fig. 5 shows that in this case there is, besides the normal increase of virus γ after 21 minutes, also an increase of virus α between 13 and 17 minutes. This increase is smaller than the increase that would have occurred in the absence of virus γ , and it is not accompanied by a simultaneous increase of the γ -titer. Calculation shows that the increase is three times smaller than that expected in the

absence of virus γ . Since just 31 per cent of the bacteria were not infected by virus γ , the increase of virus α can be ascribed to its growth in these bacteria. Evidently, only the bacteria that were infected with virus α and free of virus γ , have liberated the virus α .

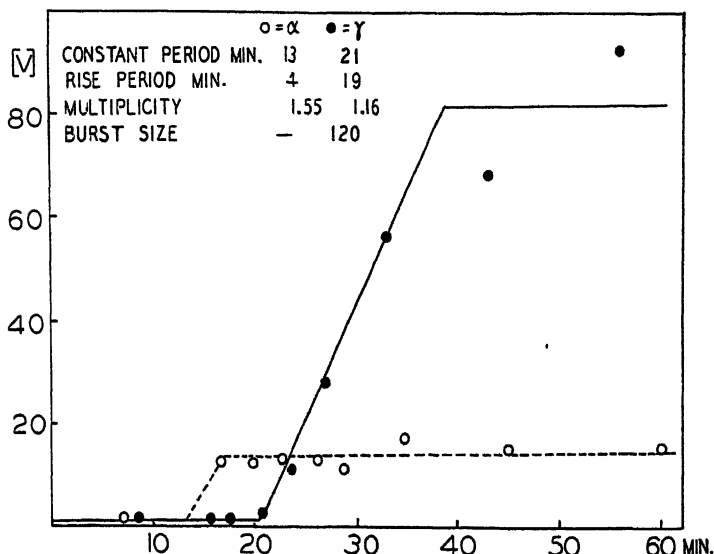


FIG. 5. Experiment no. 32. Mixed infection. Bacteria plus viruses α and γ in slight excess. \circ = virus α . \bullet = virus γ . $[V]$ = relative titer of virus.

Regarding interference we conclude that a bacterium infected with viruses α and γ will liberate only virus γ , after a latent time equal to the constant period of virus γ . No growth of virus α takes place in these bacteria.

Going back to experiment (a), we can verify that the small increase of virus α found in that case corresponds almost exactly to the liberation of virus α from the few bacteria which had adsorbed virus α and not virus γ (1.5 per cent by Poisson's formula).

(c) *Inactivation of Virus α When Adsorbed on γ -Infected Bacteria.* Let us consider a bacterium which has adsorbed both viruses α and γ , and which is plated with the bacterial strain A (sensitive to virus α only) before the liberation of virus γ has taken place. The bacterium should not produce a plaque if no liberation of virus α takes place.

In the two experiments of Figs. 4 and 5, calculation showed an initial

diminution of the titer of infective centers of virus α , when compared with the titer to be expected in the absence of virus γ . This diminution proves that a virus α particle which is adsorbed on a bacterium infected with virus γ , is actually lost. If it were liberated when the liberation of virus γ takes place, it could infect a bacterium A, and produce a plaque.

The following experiment was designed to prove this point under conditions permitting a more sensitive quantitative test. Bacteria were mixed with a large excess of virus γ and a small amount of virus α . Under these conditions practically

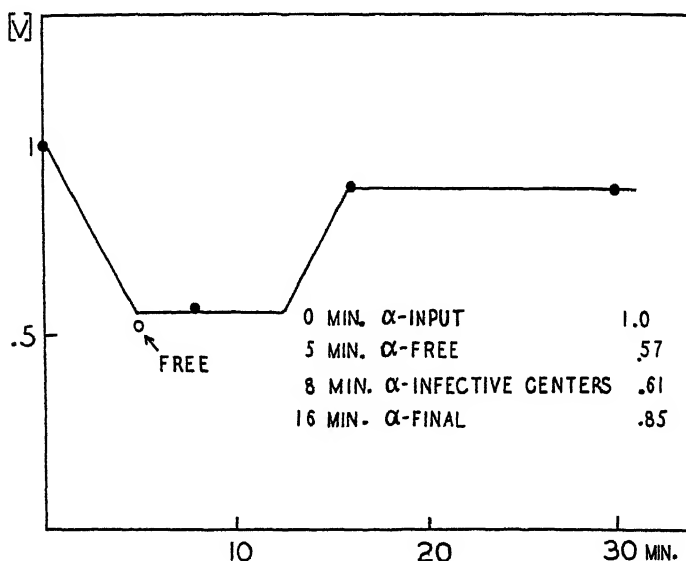


FIG. 6. Experiment no. 42. Mixed infection. Bacteria plus a large excess of virus γ and a small amount of virus α . $[V]$ = relative titer of virus α .

all the bacteria will be infected with virus γ , and any virus α which gets adsorbed should be lost. The remaining titer of α plaques should be equal to the titer of unadsorbed virus α .

The experimental results are given in Fig. 6. It shows the decrease of the titer of virus α , substantially to the amount of unadsorbed virus. Here, too, the small increase of virus α after 13 minutes is due to growth on the few bacteria which escaped infection with virus γ .

Some experiments were designed to find the limitations of the suppression of the growth of virus α by virus γ .

(d) *Mixed Infection with Virus α in Excess of Virus γ .* Experiments were done to test whether suppression takes place when virus α is present in greater amount than virus γ . In one such experiment, the multiplicity of infection was $6.3\alpha + 2.1\gamma$; in another it was $3.5\alpha + 1.4\gamma$. In both cases the suppression of virus α was evident. A small increase of virus α was quantitatively accounted for by the growth on bacteria which had not adsorbed any particle of virus γ .

(e) *Infection with Virus α Preceding Infection with Virus γ .* It might be expected that the suppression would be less complete if virus α is given several minutes start in its attack upon the bacterium. The results of a series of such experiments are listed in Table VIII.

TABLE VIII
Mixed Infection. Virus α Preceding Virus γ

Experiment	Interval of time between the infection with the two viruses	Multiplicity of infection		Increase of virus Infected bacteria	
		Virus γ	Virus α	Virus γ	Virus α
No.	minutes				
43	2	~ 3	< 1	*	1.8
76	4	7.7	4	124	3.5
40	4	4.3	1.4	210	9
74	6.5	3	3	62	105
71	7.5	6	4	17	130

* Not measured.

It is seen that suppression is complete if virus γ is added two minutes after virus α . The small increase of virus α , is again accounted for by growth on bacteria not infected by virus γ . With an interval of four minutes, the amount of growth of virus α , although small, is still too large to be compatible with suppression of α growth in all the bacteria infected with virus γ . With larger intervals of time, 6.5 and 7.5 minutes, suppression of α growth disappears rapidly; at the same time the increase of virus γ diminishes. In no case, however, is there liberation of virus α and virus γ from the same bacterium. This is proved by the fact that the increase of virus α always occurs between 13 and 20 minutes, before the beginning of the increase of virus γ .

It appears that the probability of virus α suppression declines rapidly, if the infection with virus γ occurs in the time interval between four and six minutes after infection with virus α .

(f) *Growth of Virus γ in Bacteria Multiply Infected with Virus α .* We have seen that the growth of virus γ is normal in bacteria infected by approximately equal amounts of the two viruses. It was interesting to find whether the same

was true when the bacteria were infected with many particles of virus α and only one particle of virus γ . Experiments were designed to test this point. Two one-step growth experiments for virus γ were run in parallel; in one of them the adsorption mixture received a large excess of virus α simultaneously with the virus γ ; in the other, the control, the suspension of virus α was replaced by an equal amount of broth.

It was found that an excess of virus α did not change the length of the constant period, but did reduce the total increase of virus γ to about one half the value obtained in the absence of virus α . If the reduction of the total increase is due to a reduction of the number of bacteria which liberate virus γ , the plaque counts during the constant period should also be reduced by the addition of virus α . The experiments actually show such a reduction of about 50 per cent.

DISCUSSION

The experiments described in the preceding sections establish the following facts.

The growth of each of the viruses follows the pattern of other well studied cases (7). The virus is first adsorbed by the bacterial cell. After elapse of a certain accurately reproducible time, a large amount of virus is released in a sudden burst, while the cell undergoes lysis. The time interval between adsorption and lysis varies little among the individual bacteria of a growing culture.

If the bacteria are simultaneously infected with several virus particles of the same kind (multiple infection), the results are the same, except for a somewhat increased yield of virus. However, this increased yield may be only apparent, and may be due to an underestimation of the number of infected bacteria which continue to divide for a few minutes after infection. We accept this explanation tentatively and conclude that the true yield of virus from an infected bacterium is the same in multiple infection as in single infection.

The quantitative results are summarized in Table IX.

A bacterium infected simultaneously by virus particles of both types (mixed infection), will liberate only one type, virus γ , 21 minutes after infection, as in the case of infection with virus γ alone. Virus α not only fails to grow, but the infecting virus too is inactivated. The suppression of the growth of virus α occurs even when this virus reaches the bacterial cell several minutes in advance of the suppressor, but it is then not quite complete. Some bacteria will then liberate virus α , but no virus γ will be released from these bacteria. They are lysed under the influence of virus α at the time which is characteristic for this virus

(13-17 minutes). *In no case will one bacterial cell liberate virus particles of both types.*

It should be noted that a *single* particle of virus γ is able to suppress completely the growth of virus α in any given bacterium. This is proved by the experiments on survival of uninfected bacteria, which showed that the plaque titer and the absolute number of particles of virus γ are in close absolute agreement.

The amount of virus γ liberated after mixed infection is normal, except when the cell has been infected with a great excess of virus α . In that case the yield of virus γ is somewhat reduced, because some bacteria fail to liberate either virus.

TABLE IX
Comparison of the Results for Virus α and Virus γ

	Constant period = minimum latent period	True rise period = variability of the latent period	Burst size = average yield of virus per bacterium
	minutes	minutes	
Virus α	13	3.6	142
Virus γ	21	4.2	135

We may add at this point a summary statement regarding the differences between the two viruses.

Virus α and virus γ , isolated at different times and localities, have at least one common host. When interacting separately with this host, they give plaques of different sizes. They do not induce cross immunity, i.e., the resistant secondary growth induced by either one of them is fully sensitive to the other virus. If one wishes to consider these secondary growths as strains which differ from the original one, one would say that the host range of the two viruses differs. Such close relatives as strains A, B, C may serve to differentiate between the two viruses. The physical characteristics of the two viruses α and γ are conspicuously different. This may be seen from published electron-micrographs of the two viruses (12). The pictures show striking differences both in size and structure. The difference in size is also reflected in the difference of sensitivity of the two viruses to x-rays (12, 13). Finally, differences in behavior after treatment with ultraviolet light, discussed in the next paper (20), may be mentioned. While inactivated virus γ can still suppress the growth of virus α in the bacterium and the growth of the bacterium itself, inactivated virus α shows no effects at all.

To sum up, the two viruses differ as much as any two viruses with a common host could possibly differ.

On the basis of these experimentally established facts, and of others previously found, we will now discuss the intracellular virus growth.

The following questions may be asked in this connection, some as yet inaccessible to direct experimental test, but all, probably, essential for the formulation of a comprehensive theory of virus growth:

(1) Is lysis the immediate cause of virus liberation, or is it a secondary by-effect of the infection?

(2) What determines the yield of new virus from any given cell? Is it the amount of material which is available for synthesis, or is the synthesis terminated by some other series of reactions which causes liberation and lysis after a certain time?

(3) Does multiplication proceed like that of a bacterium in a suitable growth medium, increasing from one to two to four to eight, etc., or does it proceed linearly through the intervention of some heterocatalyst from one to two to three to four, etc., or does the synthesis of all new virus occur simultaneously?

We will deal with these questions in order, proceeding from the problems with fairly direct evidence to those requiring more abstract reasoning. Although we are far from being able to construct a complete theory, the facts which have been secured help to narrow the field of speculation and suggest a scheme of interpretation of at least heuristic value.

1. Relation between Lysis and Liberation

Virus liberation and lysis occur simultaneously. Most observers have consequently pictured lysis as the immediate cause of virus liberation. The cause of lysis would then have to be sought in some by-effect of virus growth. This notion cannot explain the mechanism of virus liberation in lysogenic strains, in which lysis does not take place.

Recently, E. Cordts (14) has found evidence that lysis very probably is not the immediate cause of virus liberation. She studied a case in which virus liberation occurs in the form of a sudden burst, as in sensitive strains, but is not accompanied by lysis of the cell. In the case of this strain the cells survive infection and proceed to divide under certain conditions, namely if the medium contains more than 0.5 per cent NaCl.

This shows that virus liberation of the burst type is not the result of the lysis of the cell; the lysis appears to be rather an accessory phenomenon which may or may not accompany virus liberation. We may there-

fore restrict our discussion to the growth and liberation of virus, and regard lysis as unessential.

2. Burst Size, Latent Period

It is rather obvious that the length of the constant period is the time required by the cell for the synthesis of a standard number of virus particles, because:

(a) When the temperature of the growth experiment is changed, the constant period is changed in proportion to the growth rate of the bacteria, but the number of particles liberated per bacterium is unchanged (5);

(b) In our experiments on mixed infection, the liberation of virus γ occurred at the standard time reckoned from the infection of the cell with *this* virus, even when this infection had been preceded by infection with virus α .

The fact that the yield of virus per bacterium is nearly the same for both viruses, although the two viruses differ greatly in size, suggests that the number of particles synthesized is limited by the availability of some substrate, a definite amount of which enters into each virus particle, either of type α or of type γ .

The cycle of events which begins with infection and ends with virus liberation must be fairly independent of the bacterial division cycle, for these reasons:

(a) The length of the constant period of virus growth may be either shorter or longer than the bacterial division cycle, depending on the virus;

(b) Bacteria infected in different phases of the division cycle cannot differ much in the length of their respective latent periods, since the latent periods of the individual bacteria in the one-step growth curves vary but little. In these experiments the population of bacteria is a mixture of individuals in all phases of their division cycle.

We conclude, therefore, that the intracellular virus growth is limited by the availability of some substrate and that liberation takes place when the growth has run to completion.

3. Mechanism of Virus Growth

In order to draw conclusions about the growth mechanism itself, we must take into consideration the experiments on multiple and on mixed infection.

In multiple infection, the infection with the additional virus particles does not change, qualitatively or quantitatively, the course of events determined by one of them. This result is in conflict with the idea of a simple growth mechanism, like that of a bacterium in a nutrient medium, on the basis of which one would expect a shortening of the latent period in multiple infection. In mixed infection, the infection with virus α does not change the course of events determined by infection with virus γ .

In one respect, these two groups of experiments reveal a strikingly similar result. It would seem simplest to consider multiple infection as a special case of mixed infection, namely as the case in which the infecting strains are identical. Mixed infection then is the more general case, and as such reveals a new feature, namely the asymmetry in the relation of the two viruses to the host, virus γ being able to suppress virus α , and not vice versa.

The situation may be expressed in this way: the cellular function of growing virus is put into maximum operation by one virus particle; one virus particle saturates this cellular function. A simple hypothesis may be proposed to explain this behavior of the cell.

Hypothesis of the Key-Enzyme. The saturation may be due to the fact that among the bacterial enzymes which are necessary for virus synthesis, there is one "key-enzyme" which is completely engaged by one virus particle. Other virus particles coming later, either remain idle, or displace the first one from the key-enzyme. Thus, in multiple infection, only one particle grows; in mixed infection, virus γ displaces virus α . The key-enzyme may be just one molecule, or several, but, if several molecules, all of them must be engaged by one virus particle. When the cell divides, or rather before the cell divides, the key-enzyme must be doubled; a cell, multiple infected in this stage, would then be able to give two infected daughter cells. The incoming virus may be broken up before it engages the key-enzyme. This would explain the loss of the infecting virus α when its growth is suppressed by virus γ .

The diminution of the suppression of the growth of virus α , if this virus is given a start, is only apparently a gradual one. If the individual cell is considered, the suppression follows an "all-or-none" law: the cell either proceeds to make only virus α , or it makes only virus γ . In terms of the hypothesis of the key-enzyme this means that the virus γ particle either succeeds in displacing virus α or does not succeed. If

it succeeds, then all previous reactions tending toward synthesis of virus α are frustrated, and are replaced by the reactions leading to the synthesis of virus γ .

The key-enzyme must be a common factor in the growth of the dissimilar viruses α and γ . Obviously there must be other, specific, enzymes involved in the synthesis of each type of virus. These specific enzymes are probably those by which the indicator strains A and C differ. These indicator strains do have different enzymatic machineries, since they synthesize the "receptor-spots" for only one or the other of the viruses.

In the second paper it will be shown that experiments with virus treated with ultraviolet light are easily interpreted on the basis of the hypothesis of a key-enzyme.

The hypothesis of the key-enzyme explains the results of multiple infection as a special case of interference, which may be called "self-interference". Future experiments must show the usefulness of this concept.

It should be mentioned that the results of multiple infection might be explained in an altogether different manner, by assuming that the infecting virus particles only take part in a fast initial reaction, during which the framework for the synthesis of all the virus particles to be synthesized is laid down. If there are several similar infecting virus particles, the rate of this initial reaction may be accelerated; but, if the duration of the initial reaction is short compared to the total duration of the constant period, this acceleration would not be observable. It is necessary to assume that the infecting particle participates in the initial reaction only, since otherwise the speed of the later reactions would be influenced by multiple infection, and a change of the constant period should be observable.

It is hardly possible to elaborate this hypothesis, since it is difficult to conceive a chemical mechanism by which a virus particle can lay down a course of reactions in which it does not participate.

Interference between virus γ and virus α has no obvious interpretation in this picture. It can be expressed by saying that virus γ has a stronger directing tendency in the laying down of the framework, in fact so strong a directing force that it upsets the reactions initiated by virus α and forces the cell to follow its own directions, and indeed to follow them at a rate, as if no virus α had started specific reactions previously.

The discussion has yielded answers to the questions which we posed at the beginning. We arrive at the following picture of the growth of bacterial virus.

After adsorption on the sensitive host, the virus starts reproducing,

not autocatalytically like a bacterium in a suitable medium, but with the intervention, among other enzymes, of a key-enzyme, present in limited amount, perhaps in single unit in each cell.

The liberation of virus takes place after a definite time, when the available amount of some substrate has been used up. Interference exists both between particles of the same virus and between particles of different viruses, and is to be interpreted as a competition for an enzyme rather than for substrate.

4. Interference Phenomena in Other Viruses

(a) *Bacterial Viruses.* With our two viruses, secondary growth arising after the action of each is fully sensitive to the other virus. This is not the case when viruses are related, particularly when they are adsorbed by the same bacterial antigen or "receptor-spot." In cases where secondary growth is not truly resistant, but is a carrier of the virus (lysogenic), it will in general also be resistant to the action of related viruses. This kind of interference may be similar to that described in this paper.

The best documented instance is that described by Burnet and Lush (15). These authors worked with two related viruses, C and C', both active upon a strain SF of *Staphylococcus albus*. Virus C produces a rich secondary growth, which is lysogenic. Virus C' produces little secondary growth, which is truly resistant. The lysogenic secondary growth produced by virus C is resistant to virus C'. This resistance to virus C' is effective a few minutes after virus C has been adsorbed. Burnet and Lush assume that, in these few minutes, a true resistance has been induced in the bacterium. Bruce White (16), discussing this case in connection with similar observations of his own, suggested that the induced resistance is due rather to a blockade, by the indigenous virus, of the bacterial receptor spots. In view of our results, it seems possible that the blockade is not a blockade of the receptor spots, but of the key-enzyme. In our case, at any rate, we have unambiguous proof that the interference is not related to the adsorption of the viruses on the receptor spots.

(b) *Animal Viruses.* Hoskins (1) discovered an interesting case of interference between two strains of yellow fever virus, and a detailed experimental investigation of this has been published by Findlay and MacCallum (2).

The strains are the normal pantropic one, and a neurotropic one obtained by passage through mouse brain. If both these strains are injected either subcutaneously, intraperitoneally, or intracerebrally into a monkey, the animal shows

only the reactions characteristic of the neurotropic strain. Similar effects were observed when these strains were tested on hedgehogs and mice.

Interference between the virus of Rift Valley fever and the two strains of yellow fever, was also studied by the same investigators. The Rift Valley fever virus is serologically unrelated to that of the yellow fever and no cross immunity is induced between these two. This instance, therefore, is analogous to ours; the only similarity between the two viruses lies in the symptomatology of the induced diseases.

It was found that neurotropic yellow fever virus protects mice against the Rift Valley fever virus; the latter in turn protects monkeys against the pantropic yellow fever virus. Different hosts had to be used, because Rift Valley fever virus produces severe symptoms in mice and mild symptoms in monkeys. This introduces a limitation in the study of interference and illustrates the desirability of working with indicator hosts.

Jungeblut and Sanders (3) described a case of interference between a murine strain and a normal strain of poliomyelitis virus which are genetically related. The murine strain, of limited pathogenicity for monkeys, can protect these animals against the normal strain.

(c) *Plant Viruses*. The extensive literature concerning interference in plant viruses has been reviewed by Price (4). The present authors are not familiar with the technique of plant virus work and, therefore, are unable to fully discuss the relation of this work to their findings. However, a few remarks may be made.

In typical cases, a plant infected with one virus will, upon infection with another "related" virus, fail to develop the symptoms normally attendant upon the second infection. This failure to develop symptoms is often called "acquired immunity." In some cases (17, 18) fairly convincing evidence that the second virus actually does not grow could be adduced, and in others this is a plausible presumption. Here, then, as in our case, the growth of one virus is suppressed by the presence of another. That suppression is confined to the areas actually invaded by the first virus has also been shown in some instances (18, 19). Naturally, since the detection of this interference depends on symptom expression, it is limited to cases in which the second virus has time to develop its symptoms before the first virus destroys the tissue. Therefore, the first virus must develop symptoms more slowly than the second, or it must develop symptoms mild enough to permit expression of the second virus symptoms. For this reason, interference tests can, in most cases, only be made in one direction. This technique would not be applicable to test, for instance, whether a necrotic virus suppresses the

growth of a mottling one. It would be necessary to recourse to indicator plants sensitive only to one of the two viruses.

Acquired immunity to one virus by infection with another has sometimes been attributed to competition for common substrates and as such has been taken as a criterion of genetic relatedness. The evidence is good, that, if the viruses are known, from other experiments, to be related, they will give mutual protection. The reverse conclusion, from protection to relatedness, seems however less secure in view of our results of interference between two very dissimilar viruses. It is true that many cases of apparent lack of interference between viruses known to be dissimilar have been reported. Our case shows that interference can nevertheless be present in only one direction in the case of unrelated viruses. In those cases of plant viruses in which relatedness has been inferred on the basis of interference proved in one direction only, the correctness of the inference may be questioned.

The following scheme may be found to fit all facts:

1. Two closely related viruses will interfere in both directions.
2. Two unrelated viruses may interfere either in only one direction, or in neither direction.

It follows that:

1. Interference in both directions proves relatedness.
2. Interference in one direction is insufficient basis for assuming relatedness.

SUMMARY

1. The growth of two bacterial viruses active upon the same host is analyzed.

2. Multiple infection of a bacterium with several particles of the same virus has qualitatively and quantitatively the same effects as infection with a single virus particle.

3. Mixed infection of a bacterium with particles of both viruses results in complete suppression of the growth of one virus, while the other grows normally (non-reciprocal interference). This interference is studied in detail under various experimental conditions.

4. On the basis of these and of other results a theory of the growth mechanism of bacterial viruses is elaborated. Virus is considered to be produced with the intervention of a "key-enzyme," present in limited amount in each bacterial cell.

The results of the experiments on multiple infection are interpreted

as interference between particles of the same virus (self-interference). Self-interference and non-reciprocal interference are jointly attributed to competition for the key-enzyme.

5. The bearing of these results on other cases of interference between viruses is discussed.

Many of the experiments described in the present paper were performed in the summer, 1941, while the authors were guests of the Biological Laboratory, Cold Spring Harbor, N. Y. The authors are greatly indebted to the Long Island Biological Association and in particular to Dr. M. Demerec, Director of the Laboratory, for the hospitality extended to them. Thanks are also due Miss Edna Cordts for technical assistance.

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Essential Steps in the Enzymatic Breakdown of Hexoses and Pentoses^{1,2,3}. Interaction between Dehydrogenation and Fermentation

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1. INTRODUCTION

One of the most important ways in which products of agriculture are used is the utilization of amino acids and carbohydrates through their degradation by various fermentations brought about by microorganisms. We know what the original substances and end products are in these reactions but the task is to unfold the phase sequence of these conversions by use of the methods of enzymology.

From the time of the unforgotten discovery of Cremer (1) of glycogen formation by means of yeast juice, until the recent establishment of the fact that enzymatically synthesized starch possesses a molecular constitution (2) different from that of natural starch, the controversy whether observations made in vitro truly reflect processes and sequences proceeding in the undamaged cell, has not ceased. That the formation of the synthetic starch must be due to the action of an enzyme system significantly deficient or incomplete, was foreshadowed by measurements of the very simplest indication of a chemical reaction, namely, the heat evolved (3), which, when studied in the case of fermentations with living yeasts and with cell-free extracts, displays differences (4) of great magnitude. Accordingly, the burden of proof to the contrary is on those who disregard facts or distort interpretations.

Since the first facts (1927) concerning their similarity to the action of

¹ For a preliminary communication, see: Wirth, J. C. and Nord, F. F., *J. Am. Chem. Soc.* **63**, 2855 (1941).

² Presented before the Spring meeting of the American Chemical Society at Memphis, Tenn., April 1942.

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enzymes present in the yeast cell, the study of the intermediary phases of carbohydrate metabolism by means of different *Fusaria* added much to our information in this field (5). We succeeded recently also in the preparation of an active *Fusarium* juice (6). In spite of this work, there remained a gap and unexplained phases in the observations in the course of the breakdown, which might be regarded as decisive and common in the mechanism of alcoholic fermentation of hexoses as well as of pentoses.

The present study attempts to satisfy this demand, returning basically to the chemical identification of intermediates by utilization of undamaged *Fusaria* cells, a par excellence alcoholic fermenter, and neglecting the recently much preferred manometric methods.

2. ENZYME MATERIALS USED

The cultures used in these investigations were originally obtained from the following sources: *Fusarium lini* Bolley (FIB.) No. 5140 from the Biologische Reichsanstalt, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber; FIB. No. 309 from the North Dakota Agricultural Experiment Station, through the courtesy of Dr. W. E. Brentzel; *Fusarium oxysporum* (Fox.) No. 3221 from the Biologische Reichsanstalt, Berlin-Dahlem; Fox. No. 236 from the North Dakota Agricultural Experiment Station; *Fusarium graminearum* Schwabe (Fgra.) No. A 36-1-VIII from the University of Minnesota, through the courtesy of Dr. C. J. Eide; *Fusarium lycopersicii* (Flyco.) No. 1339a from the University of Tennessee, through the courtesy of Dr. C. D. Sherbakoff.

Stock cultures of the preceding were maintained on a nutrient medium of the following composition:

20 0 g. glucose
1 00 g. KNO ₃
1 50 g. KH ₂ PO ₄
0 75 g. MgSO ₄ ·7H ₂ O
1000 ml. water

and transferred at intervals of two weeks. Microscopic examinations were carried out to check the purity of the cultures from time to time.

3. PROCEDURES

Inoculations⁴ were made by adding 1 ml. of a uniform spore-mycelial suspension to each 50 ml. of nutrient media. This suspension was pre-

⁴ Unless otherwise noted, all experiments have been carried out with FIB. 5140.

pared by growing the fungus on a solid medium contained in a 125 ml. Erlenmeyer flask. The medium for this purpose was the same as above, supplemented by 20 g. of agar. After growth had taken place for a period of eight to twelve days, 50 ml. of sterile, distilled water was added to each flask containing the solid medium. The flask was then vigorously swirled to remove the spores and some of the mycelium from the plate.

The nutrient media were sterilized in either of two ways. If the substrate, e.g., fructose or sorbose, was found to be materially affected by heat, or, if there was any possibility that the intermediates found, might be derived from decomposition products of the substrate rather than from the substrate itself, then recourse was taken to sterilization by means of a Seitz filter. In all other cases, sterilization was carried out by streaming steam on three successive days as it was found that this method gave rise to less decomposition, as measured by products precipitable with 2,4-dinitrophenylhydrazine, than a single sterilization under pressure.

4. ANALYTICAL METHODS

Sugars with a high specific rotation such as glucose and fructose were determined quantitatively with the aid of a polarimeter. Analyses of known quantities by the optical method gave results that agreed within 1% of the amount actually present. Sugars with a low specific rotation, such as xylose and mannose, were determined by the reducing method of Lehmann-Maquenne-Schoorl (7) and ethyl alcohol by oxidation with potassium dichromate (8) after preliminary removal of the carbohydrate by means of copper sulphate-lime treatment (9). To remove any possible interfering aldehydic compounds, a small amount of silver sulphate was added prior to the distillation of the ethyl alcohol from the nutrient media.

The presence of pyruvic acid (PA) was preliminarily detected by means of an immediate positive iodoform test in the cold and a deep red coloration produced with the Lu method (10) for the quantitative determination of this substance. This compound was identified by the melting point and analysis of its 2,4-dinitrophenylhydrazone. Quantitative determinations were carried out gravimetrically with this same reagent. Recovery of known amounts of PA, in the range expected, indicated that the determination by this method was satisfactory. Hydroxylamine was detected by the color reaction of Blom (11) employing

p-bromonitrosobenzene and α -naphthol. Nitrite ions were determined by the color reaction of Griess, as modified by Blom (12) using sulphanilic acid and α -naphthylamine.

All experiments were carried out in 125 ml. Erlenmeyer flasks, 50 ml. of nutrient media being pipetted into each one. Analyses were run in duplicate for the desired constituent on an aliquot of the combined media of four to six individual flasks. This procedure was followed to compensate somewhat for possible inequalities in growth among the separate flasks. All results given have been calculated to signify the amount present in 100 ml. of nutrient medium even though a smaller quantity was actually used for the determination. Mycelial weights, however, represent the average of five to six mat weights obtained from each flask (50 ml. of nutrient medium). The mycelium was filtered from the nutrient medium by means of a porous crucible, washed with several portions of distilled water (total volume 150 ml.), dried overnight in an oven at sixty degrees, and weighed.

5. EXPERIMENTS WITH HEXOSES

In the former investigations of this series, the nitrogen required by the organism was supplied in the form of asparagine and HCN (13). The shift in the pH in the nutrient medium as observed when inorganic nitrates were used, induced the application of inorganic nitrogen sources to the investigation of the alcoholic fermentation of hexoses and pentoses. Accordingly, ammonium nitrate, potassium nitrate, and ammonium sulphate were introduced with the purpose of investigating whether a possible reducibility of a part of the nutrient medium may influence also the course of the breakdown of the carbohydrates present.

Fermentation experiments utilizing glucose, fructose, mannose, and galactose were carried out with potassium nitrate as a nitrogen source and tests were made for various conceivable intermediates. The tests indicated the absence of acetaldehyde, methylglyoxal, dihydroxyacetone, and glyceraldehyde. However, considerable quantities of PA were detected and the compound was isolated and identified as its 2,4-dinitrophenylhydrazone. Simultaneously, tests were made in the course of the fermentation for the possible occurrence of reduction products of nitrate, with the result that the presence of nitrite was established. It appears to be evident at once that the striking accumulation of PA in the presence of nitrate, in contrast to its appearance only in traces when ammonium sulphate was employed, is related to the

reduction of nitrate to nitrite. The above conclusion is found experimentally on the data given below. Experiments with glucose, fructose, and mannose were carried out with potassium nitrate and ammonium sulphate as nitrogen sources as the latter, obviously, cannot give rise to nitrite. With all three carbohydrates there was a considerable accumulation in the nitrate media but only traces in the ammonium medium. To compensate appropriately for the changes in pH of different extent due to the presence of the varied nitrogen sources, the nutrient medium had been adjusted to a pH of 5.5 with sodium hy-

TABLE 1
Relative Rates of Fermentation of Hexoses and Accumulation of PA under Varying Conditions

Day	Glucose*				Mannose*				Fructose*			
	KNO ₃		(NH ₄) ₂ SO ₄		KNO ₃		(NH ₄) ₂ SO ₄		KNO ₃		(NH ₄) ₂ SO ₄	
	Carbohydrate fermented	PA accumulated	Carbohydrate fermented	PA accumulated	Carbohydrate fermented	PA accumulated	Carbohydrate fermented	PA accumulated	Carbohydrate fermented	PA accumulated	Carbohydrate fermented	PA accumulated
	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.	g.	mg.
4	0.52	62	0.34	trace	0.53	90	0.69	trace	0.40	trace	0.30	trace
6	1.06	117	2.01	trace	2.09	194	2.36	trace	1.00	trace	0.88	trace
8	3.30	128	3.41	trace	3.49	187	3.70	trace	1.83	23	1.82	trace

* Nutrient medium: 40.00 g. hexose, 15.00 g. KH₂PO₄, 0.75 g. MgSO₄·7 H₂O, 5.00 g. KNO₃ or 3.26 g. (NH₄)₂SO₄, water to one liter, pH adjusted to 5.5.

dioxide and the phosphate concentration had been increased to 15.00 g. per liter (Table 1).

It can be seen that the rate of fermentation of mannose is slightly higher than that of glucose, while that of fructose is very much less than either of the former. If there is a basis to assume that these three sugars are fermented via a common "enol form," then the observed differences will possibly signify that the rate of conversion of the original structure to this form is really one of the controlling steps in the kinetics of fermentation.

Determinations⁵ of the cocarboxylase content revealed that there is actually more (9.5 γ of thiamin per gram) cocarboxylase in the dried

⁵ Through the courtesy of Dr. D. J. Hennessy.

mycelia of *Fusaria* when grown on a medium in which the nitrogen was supplied in the form of potassium nitrate than when ammonium sulphate was used (5.4 γ of thiamin per gram). This indicates that the accumulation of PA in the nitrate medium, as contrasted to its appearance only in traces in the ammonium sulphate medium, cannot be due to a deficiency in the carboxylase system of the organism.

Since PA was found to accumulate under conditions that may be designated as normal for the organism, as a vigorous growth was obtained on the synthetic media employed, it was deemed advisable to study the effect produced by the addition of substances known to be components of the several enzyme systems involved in alcoholic fermentation. Accordingly, the following nutrient medium

40 00 g. glucose
5 00 g. KNO₃
5 00 g. KH₂PO₄
0.75 g. MgSO₄·7H₂O
water to 1 liter

was supplemented by varying amounts of vitamin B₁ hydrochloride. (Unless otherwise stated, the above concentrations of the inorganic constituents are used in the following experiments.)

From the analytical results obtained, it is clear that the addition of this factor to the nutrient medium influences profoundly the reaction effected by the decarboxylating system of the organism, as, on the sixth day, when the amount of PA accumulated has reached a maximum, then the ratio between the amounts in the vitamin supplemented and non-supplemented media is approximately one to seven. Further, there was only one day when analyses were run, i.e., the sixth, when the amount of PA was appreciable in the vitamin containing media. It is to be noted that the maximum effect was observed by an addition as low as 50 γ per 100 ml. It was found, however, that there is a significant increase in the accumulation of PA when the concentration was reduced to the 10 γ level, the quantity of PA accumulated, when the amount in the control was a maximum, being 135, 44, and 16 mg., respectively, for the control, and the media supplemented with 10 and 50 γ per 100 ml. (Table 2). Accordingly, a transient inhibitory effect of the nitrite ions produced in the course of the reduction of nitrate appears to be compensated by the addition of thiamin.

If we compare the relative weights of the mycelia, it is apparent

that the addition of the vitamin to the nutrient medium is paralleled by a decrease in the extent of assimilation.

Since the course of the dissimilation could be so markedly changed by the addition of vitamin B₁ to the nutrient medium, it was considered of interest to determine whether the addition of other com-

TABLE 2
Effect of Varying Concentration of Vitamin B₁ on PA Accumulation
B₁ concentration in γ /100 ml.

Day	0	50	100	250	0	50	100	250	0	50	100	250
	Glucose fermented				PA				Mycelium weight			
	grams	grams	grams	grams	mg	mg.	mg	mg	mg.	mg	mg	mg
2	0 15	0 25	0 32	0 24	5	trace	trace	trace				
4	1 73	1 35	1 31	1 23	159	8	6	6	132 \pm 5	91 \pm 4	99 \pm 7	93 \pm 1
6	3 47	3 39	3 41	3 36	171	26	24	20				
8	4 09	4 07	4 07	4 08	155	trace	trace	trace	259 \pm 3	135 \pm 3	144 \pm 4	130 \pm 3

TABLE 3
Effect of Varying Concentration of Nicotinic Acid on PA Accumulation
Nicotinic acid concentration in γ /100 ml.

Day	0	10	50	0	10	50	0	10	50
	Glucose fermented			PA accumulated			Mycelium weight		
	grams	grams	grams	mg	mg.	mg.	mg.	mg	mg.
5	0 77	0 84	0 85	103	120	118			
	0 51	0 54	0 54	124	134	140			
7	3 05	3 05	3 01	119	135	160			
	2 07	2 05	2 12	108	171	172			
9	1 04	1 03	3 89	101	118	154	192 \pm 4	188 \pm 6	173 \pm 5
	3 11	3 62	3.39	162	194	168	148 \pm 3	165 \pm 10	132 \pm 4

pounds which are related to integral parts of some members of the enzyme system operative in alcoholic fermentation would likewise be effective. Accordingly, the glucose nutrient medium was supplemented by nicotinic acid. The results of typical experiments under these conditions are given below (Table 3).

From the data here presented, it can be seen that the nicotinic acid

has no influence on the rate of disappearance of glucose, whereas the amounts of PA accumulated in the latter stages of the experiment are increased in some cases by 15, 30, and 50% above those amounts which were obtained without the addition of nicotinic acid. Accordingly, there appears to be a basis for the assumption that the presence of nicotinic acid in the nutrient medium makes it possible for the organism to more readily synthesize the dehydrogenating enzymes. The possibility of an augmented dehydrogenase effect may result in an additional reduction of the acceptor—nitrate to nitrite ions whose inhibitory effect on carboxylase was demonstrated in dried yeast preparations (14).

TABLE 4
Effect of Varying Concentrations of KNO₃ on PA Accumulation
Nitrate concentration in grams/1000 ml.

Day	2 00	5 00	8.00	2 00	5.00	8.00	2.00	5.00	8.00
	Glucose fermented			PA			Mycelium weight		
	grams	grams	grams	mg.	mg.	mg.	mg.	mg.	mg.
3	0.44	0.35	0.36	76 = 17 7%	83 = 24.3%	91 = 25 9%			
5	2.24	2.48	2.87	123 = 0 56%	202 = 0.83%	249 = 0.88%			
7	3.76	3.78	3.92	93 = 0.25%	202 = 0.57%	271 = 0.71%	206±3	239±7	265±2

If one could assume that a hypothetical phase sequence in the degradation, by means of artificial enzyme systems, from hexoses to alcohol is also followed in the case of living *Fusaria*, then for an accumulation of PA to occur, some hydrogen acceptor or acceptors other than acetaldehyde must be operative so that a corresponding oxidation can proceed at a faster rate than the subsequent steps. The assumption of some other hydrogen acceptors was verified by the finding of both nitrite and hydroxylamine as reduction products of nitrate and also by the observation that there is a relationship between the amount of PA accumulating, both in absolute quantities and percentage yields (if we are to assume that all of the glucose were converted to PA) and the nitrate concentration (Table 4). From the data recorded, it can be seen that increasing amounts of nitrate also accelerate the rate of glucose consumption and enhance the fungus growth.

An accumulation of PA without resort to trapping agents or arti-

ficial enzyme preparations may lead one to suspect that the equilibrium and order in the phase sequence of degradation in the living organism might have been disturbed. It was, therefore, necessary to determine the amounts of alcohol produced under our experimental conditions.

TABLE 5
Glucose Fermentation

Day	Glucose fermented	PA	Et OH
	<i>grams</i>	<i>mg.</i>	<i>grams</i>
5	1.16	140	0.28
7	2.84	151	0.94
9	3.78	155	1.19

TABLE 6
PA Accumulated When Various Strains Were Employed

Day	Fgra		FIB, N. Dakota		Fox, N. Dakota		Fox, Dahlem		Flyco	
	Glucose fermented	PA	Glucose fermented	PA	Glucose fermented	PA	Glucose fermented	PA	Glucose fermented	PA
5	0.15	45	1.59	146	0.86	72	1.19	97	0.82	105
7	0.72	63	3.25	154	2.17	72	2.81	88	1.90	122
9	0.85	70	3.94	178	3.48	72	3.79	85	2.87	119

Glucose in grams, PA in milligrams.

TABLE 7
Galactose Fermentation

Day	Galactose fermented	PA
	<i>grams</i>	<i>mg.</i>
4	0.75	29
6	1.92	49
8	3.31	42

In the table above are recorded the amounts of glucose fermented, PA accumulated and alcohol formed (Table 5).

From these data, it appears justified to conclude that there has been no deviation in the phase sequence of the dissimilation, and that, despite the accumulation of an intermediate, there has been no other

than the customary production of the established end products, namely, ethyl alcohol and carbon dioxide.

To determine whether this accumulation is a characteristic of our particular strain of FIB., fermentations of glucose were carried out with additional types of *Fusaria* indicating practically the same magnitude of PA accumulation in all cases (Table 6).

To supplement our investigation on the hexoses, a 4% solution of d-galactose was fermented. The rate at which galactose is fermented is virtually the same as for glucose. However, the amount of PA accumulated is significantly lower (Table 7).

6. EXPERIMENTS WITH PENTOSE

It will be recalled that *Fusaria* are able to degrade pentoses, and that in the case of xylose, at least, considerable quantities of alcohol are formed. Contrary to the breakdown of pentoses by distillation in alkaline medium (15), only little is known concerning the intermediate phases of their alcoholic breakdown. It was of great interest, therefore, to determine whether PA could be found during the fermentation of this class of carbohydrates as well.

Employing a nutrient medium with a d-xylose concentration of 3%, it was found that PA did accumulate but to a somewhat less marked extent. This fact is easily understandable, if we assume that from each pentose molecule only one PA molecule can be formed on the basis of a C_3 - C_2 split. Such a split is not unlikely as we have succeeded in actually isolating a C_3 compound although efforts to trap or isolate glycolaldehyde (GA), which may be regarded as the C_2 moiety, have been thus far unsuccessful. An inability to trap GA in the course of pentose fermentation could be related to the consideration that the rate of the enzymatic conversion of the GA formed from the pentoses within the cell is higher than are the possible rates of diffusion out of the cell or of permeation of added GA into the cell. This, in fact, served as a weak carbon source.

As in the case of glucose, the effect of varying amounts of potassium nitrate on the accumulation of PA was investigated, the results being recorded in Table 8.

From these data, it can be seen that the nitrate concentration does affect the amount of PA accumulating. In addition, we see that the quantity of alcohol remains practically constant beyond a certain amount of nitrate. Furthermore, the influence of the concentration

of the nitrogen source on the rate of xylose consumption is marked only in the range of concentration between two and five grams per liter. It should be further noted that in the case of xylose there is practically no increase in mycelial weight with increasing nitrate concentrations as contrasted with the marked increase when glucose was the carbon source (cf. Table 4).

It had been previously noted that nitrite ions were being formed during the course of the fermentation of glucose. As a possible explanation for the accumulation of PA, we have assumed an inhibitory action of nitrite on the carboxylase system present in *Fusaria*. Therefore, if, as with hexoses, a nitrogen source such as ammonium sulphate is employed, there should be no marked accumulation of PA. Also,

TABLE 8

Effect of Increasing Amounts of KNO₃ on Accumulation of PA and Alcohol Formed from Pentose

Nitrate concentration in grams/1000 ml.

Day	2.00	5.00	8.00	2.00	5.00	8.00	2.00	5.00	8.00	2.00	5.00	8.00
	d-Xylose fermented			PA accumulated			Ethyl alcohol			Mycelium weight		
	grams	grams	grams	mg	mg	mg	grams	grams	grams	mg	mg.	mg.
7	1.43	1.70	1.67	16	41	33	0.32	0.37	0.36			
9	2.09	2.28	2.26	23	37	29	0.48	0.53	0.52			
12	2.65	2.80	3.00	20	32	18	0.54	0.59	0.62	102±2	105±8	127±7

since it has been shown (16) that when ammonium nitrate is present as a sole nitrogen source in culture media for *Fusaria*, the nitrate ions are preferentially used. Therefore, to attempt to test experimentally this hypothesis, parallel experiments were run with various inorganic nitrogen sources. Ammonium sulphate, potassium nitrate, and ammonium nitrate were present in the nutrient media in such concentrations that the quantity of nitrogen in the three was identical. In the case of ammonium nitrate, the amount of this substance was calculated on the basis of nitrogen in the nitrate radical alone for the reason previously mentioned. A nutrient medium of the same composition as in Table 1 was used, but with a xylose concentration of 3%, to compensate for the pH changes mentioned in connection with this experiment (Table 9).

From the data recorded, it can be seen that with pentoses as well as

with hexoses (cf. page 146), the accumulation of PA is dependent on the formation of nitrite ions as with ammonium sulphate only a trace of this intermediate was detected. On the other hand, the amount of PA accumulated was of the same magnitude when both potassium and ammonium nitrate were used as nitrogen sources. However, the ammonium nitrate gave rise to slightly higher percentage yields of ethyl alcohol from the xylose. Ammonium sulphate, on the other hand, is a less satisfactory source of nitrogen with respect to both rate of disappearance of xylose and the final formation of ethyl alcohol. It was during the progress of this experiment that the course of the

TABLE 9
Pentose Fermentation Related to Nitrogen Source and Biotin

Day	5.00 g. KNO ₃			5.00 g. KNO ₃ , 2.5 γ Biotin			3.26 g. (NH ₄) ₂ SO ₄			3.96 g. NH ₄ NO ₃		
	Xylose fermented	Ethyl alcohol	PA	Xylose fermented	Ethyl alcohol	PA	Xylose fermented	Ethyl alcohol	PA	Xylose fermented	Ethyl alcohol	PA
	g.	g.	mg.	g.	g.	mg.	g.	g.	mg.	g.	g.	mg.
7	1.81	0.35	29	1.63	0.39	29	1.46	0.29	trace	1.64	0.38	38
9	2.63	0.56	34	2.10	0.49	29	1.78	0.37	trace	2.08	0.53	36
12	2.89	0.59	16	2.88	0.57	14	2.74	0.41	trace	2.74	0.59	10

Mycelium weight on 12th day

138 ± 3 mg.

169 ± 13 mg.

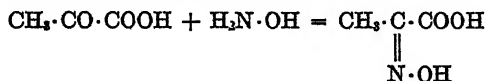
285 ± 9 mg.

166 ± 6 mg.

Nutrient medium: 30.00 g. xylose, 15.00 g. KH₂PO₄, 0.75 g. MgSO₄·7 H₂O, pH 5.5.

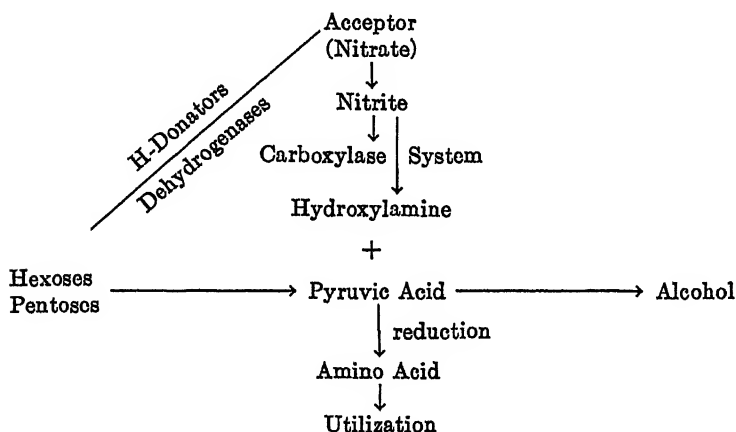
nitrate → nitrite reaction was further investigated and it was established that the reduction of nitrate proceeds beyond the state of nitrite insofar as we obtained a positive hydroxylamine test, which, however, turned negative after the fourth day.⁶ Experiments with added nitrite and pyruvate appeared, therefore, to be useless.

⁶ According to V. Meyer and A. Janny, *Ber.* 15, 1527 (1882), pyruvic acid forms with hydroxylamine isonitrosopropionic acid



which may be further reduced to alanine. In this case, in biological systems, a

The fact that the nitrates present in the media were easily reduced as far as hydroxylamine indicates that they may serve as an indirect acceptor for the hydrogen loosened by the dehydrogenases and supplied by one or more hydrogen donors. The nitrite, in turn, serves as a temporary inhibitor of the carboxylase system—furnishing a means to disclose a member of the phase sequence of carbohydrate breakdown. The limited accumulation of PA and the permanent functioning of the carboxylase system involves a continuous oscillation between the nitrite-inhibited and the free enzyme, indicating that the nitrate—nitrite reaction proceeds at a faster rate than the reduction of the latter to hydroxylamine. The mechanism may be represented schematically as follows:



This also invalidates earlier considerations of Meycrhof (17) with regard to the rate of transformation of intermediates in the living cell.⁷

It appears, therefore, to be justified to emphasize that in the case of *Fusaria*, according to the nitrogen source present, there exists an

cycle of utilization of nitrate by the cell is closed. It may be noted here that in experiments with *Fusaria* which were conducted with amino acids as sole carbon and nitrogen sources, the formation of a keto acid from alanine was detected by means of the Lu method.

⁷ Cf. Goethe, *Faust*:

Was diese Wissenschaft betrifft,
Es ist so schwer, den falschen Weg zu meiden,
Es liegt in ihr so viel verborgnes Gift,
Und von der Arznei ists kaum zu unterscheiden.

organic interaction between the dehydrogenating enzyme system and the zymatic system. The fact that fermentable sugars and sugars designated not long ago as non-fermentable have exposed the same key substance as an intermediate, in the course of their alcoholic fermentation, seems to us all the more significant because in the latter case it leads also to the realization of the second component of the C_5 chain which may be GA.

As preceding work of this series (l. c.) had shown that *Fusaria* could enzymatically degrade both d- and l-arabinose, a search for PA as an intermediate in the dissimilation of these compounds as well as of d-ribose⁸ was undertaken. In the case of both d-isomers, the presence of a keto acid was indicated when the Lu test was employed. However, the extent of accumulation of this acid was such that no derivative

TABLE 10
Fermentation of l-Arabinose

Day	<i>l</i> -Arabinose fermented	PA accumulated
	<i>grams</i>	<i>mg.</i>
4	0.17	trace
6	0.55	trace
8	1.06	trace
10	1.47	43
12	2.00	115

could be isolated for purposes of identification. With l-arabinose, on the other hand, in preliminary experiments, we obtained large amounts of PA. The results of a typical experiment are recorded in table 10.

From the data presented, it can be seen that the rate of dissimilation of l-arabinose is much slower than that of d-xylose, while the amount of PA is larger and appears much later in the course of the fermentation of the former. This striking difference between the d- and l-forms is all the more remarkable if we consider that of the hexoses both glucose⁹ and mannose are closely related to arabinose. With both of these hexoses, large quantities of PA accumulated while with the corresponding pentoses it was the l- and not the d-isomer which gave rise to the PA in quantity.

⁸ Obtained from Dr. G. Henning, Ltd., Berlin-Tempelhof.

⁹ l-Glucose, according to unpublished observations of R. C. O'Connor, cannot serve as a sole carbon source for *Fusaria*.

That d-ribose may serve as an excellent sole carbon source for *Fusaria* is shown by the accompanying photomicrograph (Fig. 1). The fact, however, that the test for the intermediary keto acid was extremely weak and no traces of ethyl alcohol were obtained indicates the possibility of an attack on this molecule resulting from a dehydrogenation rather than from alcoholic fermentation.



FIG. 1 FIB grown on a 2% d-ribose, KNO_3 nutrient medium
Magnification 400 \times .

7. EXPERIMENTS WITH BIOTIN

Biotin has been reported in the literature as a growth factor for many microorganisms. One species of *Fusaria*, i.e., *F. avenaceum* would not grow at all unless this factor was added to the nutrient medium (18). It was considered, therefore, of interest to determine whether the addition of this substance to our nutrient media would have any significant effect on the growth and metabolism of the organism. Accordingly, the potassium nitrate nutrient medium used in the experiments recorded in Table 9 was supplemented by 2.5 γ of biotin methyl ester per liter. The results so obtained are set forth in Table 9, Column 2. As represented by the mycelial weights, there was only a very slight

effect. This may be due to our method of inoculation (*q'*) as it has been shown that agar contains (19) biotin and we took no special pains to insure the complete removal of the growth factor from the blank, or FIB. itself may be able to readily synthesize it as do other micro-organisms (20). No explanation is offered, however, for the observation of decreased xylose consumption in the biotin supplemented medium.

8. EXPERIMENTS WITH DIMEDON

It is evident, from the fact that, since PA can be accumulated in the course of fermentation of both hexoses and pentoses, the rate of the reaction: precursor \rightarrow PA must be greater than the rate which governs the following transformation: PA \rightarrow acetaldehyde. Acetaldehyde had been reported as an intermediate in the fermentation of carbohydrates by *Fusaria*. However, the origin of the compound was never established conclusively. As to the possibility of different sources of acetaldehyde in the course of the degradation to and from ethyl alcohol, attention should be paid to the finding (21) that the bisulphite addition compound of PA can be decarboxylated by yeast and yeast preparations with the resulting formation of the acetaldehyde-bisulphite compound. This fact always leaves open the question that when acetaldehyde is blocked by bisulphite the trapping agent is either combining with the PA first or with the acetaldehyde itself which in fermentations with *Fusaria* could come from ethyl alcohol also.

It was found possible to trap acetaldehyde by bisulphite in fermentations of glucose with *Fusaria*. For this experiment the following nutrient medium was used:

40.00 g. glucose
3.26 g. $(\text{NH}_4)_2\text{SO}_4$
2.50 g. KH_2PO_4
30.00 g. CaSO_4^*
0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
1000 ml. water
pH 7.0

* Wet weight, freshly precipitated (22).

the carbohydrate and inorganic constituents being mixed after each had been sterilized separately. One hundred ml. of medium were contained in a 250 ml. Erlenmeyer flask and each flask was inoculated with 2 ml. of a spore-mycelial suspension. Ammonium sulphate, a

lowered phosphate concentration, and a pH of 7 were chosen for the following reasons. With ammonium sulphate as a nitrogen source, the medium becomes more acid due to the preferential utilization of the ammonium ions. As a consequence of this shift in pH, the insoluble calcium sulphite will be slowly converted to the soluble bisulphite. To make such a change more readily possible, it is necessary to have only a weakly buffered system—therefore, the lowered phosphate concentration. The pH was adjusted to 7.0 as this approximates the pH of a suspension of calcium sulphite in distilled water (determined experimentally) and as it was found by previous experience that soluble sulphites prevent the growth of *Fusaria*. The flasks were frequently shaken during the fermentation and at the end of three weeks, the *Fusaria* and precipitate were removed by filtration. One gram of glucose per 100 ml. had been fermented. Three hundred ml. of the filtrate were strongly acidified with sulphuric acid and distilled with an efficient condensing system. The distillate was collected below the surface of ice cold water. After about 80 ml. had been collected, no more aldehyde was coming over as was evidenced by a negative Tollens test. The sulphurous acid was then removed from the distillate by addition of hydrated calcium chloride and 200 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid was added. After standing overnight in the refrigerator, the precipitate was filtered off, dried, and weighed. 315 mg. of the 2,4-dinitrophenylhydrazone of acetaldehyde were thus obtained which corresponds to 62 mg. of acetaldehyde.

However, this method, even though successful, still leaves open the previously raised question of origin. To obviate this difficulty, experiments were carried out with dimedon as this reagent reacts only with aldehydes and not with ketonic compounds. As it had been shown that the amount of acetaldehyde remaining in solution in the presence of an excess of dimedon is greatly dependent on pH (23), the following nutrient media (all saturated with respect to dimedon) were used for this series of experiments (Table 11).

From the data presented and the fact that the pH, at least during part of these experiments was at the very optimum for the trapping of acetaldehyde, it is evident that acetaldehyde itself does not accumulate during the course of an alcoholic fermentation with *Fusaria* and that this non-accumulation, in the presence of large quantities of carbohydrate is independent of the form of the inorganic nitrogen source.

From this fact the conclusions can be drawn that the reaction: acetaldehyde plus hydrogen \rightarrow ethyl alcohol must be extremely rapid and that any deviation of determined alcohol values from calculated values seems to be independent of the dehydrogenation of the alcohol formed (0.89 g. were present on the tenth day in medium "1" and its presence was likewise qualitatively checked in media "2" and "3") in the course of carbohydrate breakdown, at least while carbohydrate was still present. This conclusion was further confirmed (24) by experiments in the course of studies of the chemistry of the dehydrogenation of alcohols

TABLE 11
Experiments with Dimedon

Day	Medium 1*		Medium 2*		Medium 3*	
	Glucose fermented	pH	Glucose fermented	pH	Glucose fermented	pH
	<i>grams</i>		<i>grams</i>		<i>grams</i>	
6	0.59	4.0	0.70	4.1		
8	1.20	4.0	1.48	4.1		
10	2.52	4.3	2.31	4.2		
14					3.59	3.6

* Consisting of:

40.00 g. glucose	40.00 g. glucose	40.00 g. glucose
15.00 g. KH_2PO_4	15.00 g. KH_2PO_4	15.00 g. KH_2PO_4
5.00 g. KNO_3	3.96 g. NH_4NO_3	3.26 g. $(\text{NH}_4)_2\text{SO}_4$
0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.75 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
water to one liter, pH 4.0	water to one liter, pH 4.0	water to one liter, pH 5.5

which showed that acetaldehyde can be trapped by dimedon when ethyl alcohol serves as a sole carbon source for the fungus.

Attempts to trap acetaldehyde with dimedon from solutions of high concentration (4%) of d-galactose, d-fructose, d-mannose, d- and l-arabinose, and d-xylose, and d-ribose (2%) with potassium nitrate as nitrogen source were without success, indicating that acetaldehyde is not even slightly accumulated in the dissimilation of any of these compounds.

However, in contrast to these results, from a 4% l-sorbose solution a copious precipitate of the dimedon derivative of acetaldehyde was obtained from 500 ml. of nutrient. This precipitate began to appear in from four to six days. On the eleventh day, both precipitate and

mats were removed from the nutrient medium by filtration. The former weighed 0.30 g. and was identified by melting point and analysis.

As PA accumulation is markedly decreased by the addition of vitamin B₁ to the nutrient medium, it was thought that possibly the addition of this factor would cause an accumulation of acetaldehyde by effecting a more rapid decarboxylation of its precursor, PA, and that the former compound could then be trapped. Experiments designed to test this assumption were negative with the glucose—potassium nitrate nutrient medium.

9. DISCUSSION

Data attempting to elucidate the enzymatic mechanism and stoichiometric relations of carbohydrate breakdown have been generally, so far, based on observations drawn from the action of yeasts, yeast preparations and juices, and muscle preparations upon hexoses. However, in recent years, we succeeded in throwing light on some details of the mechanism of alcoholic fermentation of hexoses as well as of pentoses by means of different living *Fusaria*.

Besides being not only KCN insensitive, *Fusaria* are able to utilize this compound as a nitrogen source, and do not appear to enzymatically utilize organic or inorganic phosphates during the first phases of carbohydrate dissimilation. On the other hand, the existence of a common and decisive phase, and what this phase may be preceding the terminal ethyl alcohol formation from hexoses as well as pentoses, had not been demonstrated heretofore. The tool to the solution of this problem was an appropriate application of nitrogen sources, connected with observations on the path of their chemical changes. Two findings were made simultaneously. Hexoses, as well as the pentoses d-xylose and l-arabinose supplied us in the course of their fermentation with isolatable amounts of PA and simultaneously the nitrate present in the nutrient media was reduced to the stage of hydroxylamine, in distinction to ammonium sulphate which cannot be so changed. On the other hand, the addition of vitamin B₁ or nicotinic acid caused a more rapid disappearance or gave rise to additional amounts, respectively, of the PA so formed.

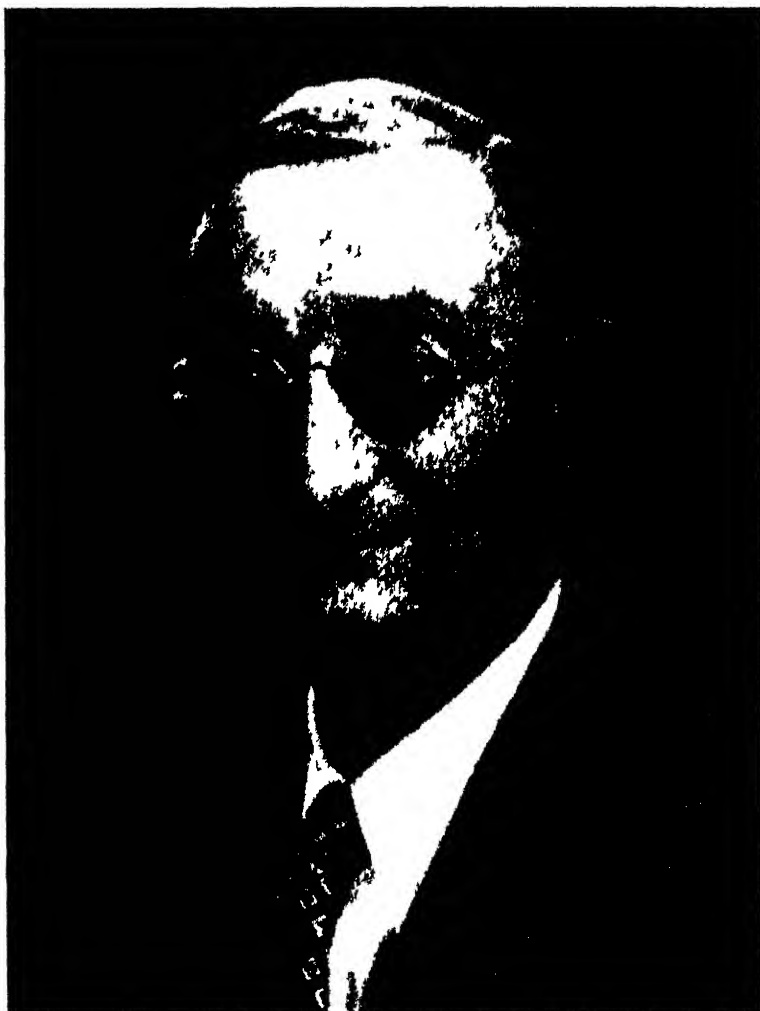
Consolidating results of earlier investigations with the fact that, in the course of fermentations by *Fusaria*, phosphoglyceric acid was not (25) isolated in the presence of fluoride, this phase sequence, inter-

a nutrient medium in which the nitrogen is supplied in the form of potassium nitrate than when grown on similar media in which the potassium nitrate has been replaced by ammonium sulphate.

8. No acetaldehyde is trapped in the course of the alcoholic fermentation of hexoses or pentoses with dimedon so long as carbohydrates are present in the media.

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Dr. Ross Aiken Gortner

1885 - 1942

Ross Aiken Gortner

1885-1942

Death claimed one of America's distinguished scholars, Ross Aiken Gortner, on September 30, 1942. Born fifty-seven years ago in Nebraska, and educated at Nebraska Wesleyan (B.S., 1907), University of Toronto (M.S., 1908), and Columbia University (Ph.D., 1909), Dr. Gortner came to the University of Minnesota in 1914 and remained with that institution until the close of his life. While first associated there with Dr. F. J. Alway, in the Division of Soils, he was transferred to the Division of Agricultural Biochemistry in 1916. When Dr. Thatcher became Dean and Director of the Department of Agriculture in 1917, Dr. Gortner succeeded him as Chief of the Division of Agricultural Biochemistry and occupied that position through the intervening quarter of a century.

As chief of that division, Dr. Gortner exhibited many and varied interests. A primary consideration in all of his teaching and research was to place both of these functions upon a sound basis of fundamentals. The large group of graduate students who were attracted here found themselves subjected to rigorous schooling in the sciences upon which the broad and rather inclusive science of biochemistry is based. Their minds, as well as that of their advisor, were continually directed to the search for the underlying physical or biological law or principle upon which the phenomenon under observation depended.

Probably Dr. Gortner found more satisfaction in this program of training advanced students than in any other personal achievement. He never spared his energies in counseling and advising, developing new methods or devising new equipment for them to employ. His colleagues in the Division found themselves swayed by the same general ideals and his influence was thus extended through other groups of students who were not actually numbered among his advisees, including those who were qualifying for industrial work. The soundness of his policies was recognized by the Graduate School of the University of Minnesota which made him Chairman of the Graduate Group Committee in Agriculture. It is evident that he contributed substantially to the whole

program of graduate study throughout the Department of Agriculture and other departments of the University, and his counsel was frequently sought in establishing institutional policies.

Through the period of his active researches, extending over approximately thirty-five years or more, Dr. Gortner covered a diversity of fields, of which only the more prominent can be mentioned here. These included the melanins, plant sap properties as a function of species and habitat, constitution of the proteins, colloidal systems, state and role of water in living and other materials, relation of flour protein properties to baking strength, chemistry of wood, and certain aspects of wood processing. His researches resulted in the publication of upwards of 350 journal papers. In addition he was the author of "Outlines of Biochemistry," originally published in 1929, and largely rewritten and expanded as the second edition that was published in 1938.

Naturally Dr. Gortner's services were in great demand at many times and places, and he gave most generously of his energy and strength in response to these demands. He served as the George Fisher Baker lecturer at Cornell University in 1935-36, and his lectures were compiled and published in book form two years later. He contributed extended articles to several symposia and monographs and was one of the editors of the records of the late J. Arthur Harris which were published in book form in 1936. The list of committees of scientific organizations on which he served in the past twenty-five years is very extended, and the services which he rendered were varied, and of a very substantial nature. Included among these were his contributions to the activities of the committee on Organic Chemical Nomenclature of the American Chemical Society and of the American Society of Biological Chemists, and the committees on Biochemical nomenclature, Chemistry of Proteins, Colloid Science, and Organic Chemical Nomenclature of the National Research Council. At various times he served as Councilor at large of the American Chemical Society, Chairman of the Division of Biological Chemistry, and also of the Division of Colloid Chemistry of that society; he was assistant editor of Chemical Abstracts continuously since 1914, and also was Associate Editor of the Journal of the American Chemical Society, and of the Journal of Physical Chemistry for extended periods.¹

It was only appropriate, thus, that many honors and distinctions came

¹He was one of the co-founders of the Archives of Biochemistry and devoted enthusiastically much of his time and experience to this new venture.

to him. The honorary degree of Doctor of Science was conferred on him by Lawrence College in 1932; he was made a member of the National Academy of Science in 1935; he was a member of Phi Kappa Phi, Gamma Sigma Delta, Alpha Chi Sigma; he was national president of Phi Lambda Upsilon from 1921 to 1926, and was serving as president of Sigma Xi at the time of his passing. In 1942 he was the recipient of the Thomas Burr Osborne medal, conferred "for distinguished contributions in cereal chemistry" by the American Association of Cereal Chemists.

In addition to these professional attributes, Dr. Gortner was a real friend to many people here and abroad. His home in St. Anthony Park was always open to his students and other associates where kindly interest, aid in times of difficulty, counsel concerning technical matters, and a charming companionship was uniformly and generously extended. Those who enjoyed the privilege of association with him have been singularly fortunate. His virile interest in science, his passion for truth and honesty in all things, his insistence upon fair dealing, and his joy in accomplishments of his colleagues and students permeated the institution of which he was a part, and now stems out into the far reaches of the world where his students are to be found.

C. H. BAILEY.

The Stability of the Prothrombin of Lyophilized Human Plasma

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In 1935, Florsdorf and Mudd (1) stated that plasma, dried by the lyophile technic and subsequently restored to the liquid state with distilled water, contained less prothrombin than did the original plasma. It was suggested that the increased alkalinity of the restored material as compared with unprocessed plasma (caused presumably by the loss of carbon dioxide during the desiccation procedure) was responsible for the partial inactivation of the prothrombin. Later, Strumia and McGraw (2) claimed that "plasma dried from the frozen state and restored with pyrogen-free distilled water . . . shows almost complete loss of prothrombin," but that restoration of such plasma with distilled water saturated with carbon dioxide results in a "preservation of prothrombin in the neighborhood of 55 per cent of normal." Strumia also suggested that the high pH of plasma restored with distilled water (reported by him to be 7.8 to 8.1), resulting from the loss of carbon dioxide and the use of sodium citrate as an anticoagulant, was responsible for the loss of prothrombin. In a later paper (3) this same investigator reported that the average prothrombin content of plasma restored from the dry state with distilled water (pH 8.2 to 9.3) was 15 to 20 per cent of normal. When plasma was lyophilized and restored with cold distilled water saturated with carbon dioxide, the prothrombin level was found to have decreased from an original level of 80 per cent to a level of 50 to 60 per cent. However, when a dilute citric acid solution (0.1 per cent) was used for restoration, the prothrombin content of the restored plasma (pH 7.85) was found to be 67 per cent of normal, as compared with a value of 65 to 70 per cent prior to desiccation. This latter result indicates a complete recovery of prothrombin activity. Tocantins (4) found that a loss of prothrombin activity resulted when citrated or oxalated plasma was exposed to an air current. A simultaneous increase of pH occurred.

The prothrombin activity could be brought back essentially to normal by treatment of the aerated plasma with carbon dioxide. Tocantins did not believe that this effect of carbon dioxide was due entirely to its effect on pH. Bubbling oxygen through the aerated plasma did not influence the results obtained. Quick (5) repeated Tocantins' experiments, and reported that aeration of dog and rabbit plasma at 38°C. did not cause a loss of prothrombin activity. When human plasma was aerated, no loss of activity was found at room temperature, but a definite loss occurred at 38°C. Quick was unable to restore the prothrombin time of this aerated human plasma to normal with carbon dioxide, and concluded that the decrease in prothrombin observed at 38°C. was due to some unknown oxidation reaction.

EXPERIMENTAL RESULTS AND DISCUSSION

Stability of Prothrombin to the Lyophile Technic. Serial dilutions of a sample of citrated human plasma (pH 7.2) were made, using physiological sodium chloride solution as a diluent. Clotting times then were determined at 37–38°C. by the method of Quick (6), using two different rabbit brain thromboplastin preparations. Samples of this same plasma were desiccated by the lyophile technic (1). After restoration with distilled water alone, and with carbon dioxide and distilled water, serial dilutions again were made. The clotting times of the restored plasma samples were determined, using the thromboplastin preparations previously employed. The values found are recorded in Table I.

The results obtained in this experiment indicate that the lyophile technic does not cause an appreciable loss of prothrombin. The "loss" reported by other investigators is only apparent, and appears to be explained by the abnormal pH values obtained when lyophilized plasma is restored with distilled water or with improperly adjusted mixtures of carbon dioxide and water. The effect of pH on the clotting times obtained with the Quick procedure will be indicated in a later section of this paper.

In another experiment, clotting times were determined for various dilutions of a sample of citrated plasma. This plasma then was desiccated by the lyophile technic, after which it was stored at 5°C. Samples were restored with carbon dioxide and water after 20 days, and after 45 days respectively. The data obtained are tabulated in Table II. The averages of the three clotting times for each plasma dilution were plotted against the plasma dilutions (Fig. 1). The figures given in

TABLE I

Clotting Times (Seconds) of Various Dilutions of Citrated Human Plasma as Determined by the Quick Method

	Per cent plasma	Original liquid plasma, pH 7.2	Plasma restored with water and carbon dioxide, pH 7.25	Plasma restored with distilled water, pH 9.15	Apparent prothrombin level of plasma restored with distilled water*
Plasma No. 1	100	16.9	16.4	34.8	<i>per cent</i> 24.5
	80	18.5	17.7	34.1	25.0
Thromboplastin No. 1	60	21.2	20.8	31.1	29.0
	40	25.8	24.5	37.2	22.0
	20	40.5	44.2	59.0	13.0
	10	86.4	83.0		
Plasma No. 1	100	15.4	15.5	22.1	49.5
	80	16.8	17.6	22.3	48.5
	60	19.8	20.6	26.1	39.5
Thromboplastin No. 2	40	25.7	28.1	36.6	28.0
	20	48.1	55.5	79.3	11.0
	10	118.0			

* Taken from a standard curve prepared by plotting the values obtained for the original liquid plasma; the prothrombin level of the original undiluted plasma is regarded as 100 per cent.

TABLE II

Clotting Times (Seconds) of Citrated Human Plasma at Various Times after Desiccation by the Lyophile Technic, as Determined by the Quick Method

Per cent plasma	Before drying, pH 7.2	20 days after drying,* pH 7.25	45 days after drying,* pH 7.25
100	18.1 (99)†	17.7 (105)†	18.7 (91)†
80	19.0 (85)	19.4 (81)	19.7 (78)
60	20.8 (68)	21.9 (60)	22.2 (59)
40	25.6 (43)	27.2 (38)	27.3 (37)
20	36.2 (21)	33.9 (24)	44.0 (16)
10	72.5 (10)		

* Lyophilized plasma held at 5°C.; restored with carbon dioxide and water.

† Figures in parentheses were obtained from the curve given in Fig. 1. Theoretically, they should correspond to the figures given in the left hand column ("Per cent plasma") of this table.

parentheses in Table II were obtained from this average curve, and represent experimentally determined values for "per cent prothrombin," where it is assumed that the prothrombin level of the undiluted plasma was 100 per cent. It will be observed that, within the limits of error of this type of analytical procedure, the prothrombin level of the lyophilized plasma remained essentially unaltered for a period of at least 45 days. The effect on the prothrombin level of lyophilized human plasma of storage at various temperatures for long intervals of time will be reported at a later date. On the basis of preliminary unpublished data, we predict that no striking changes will be found.

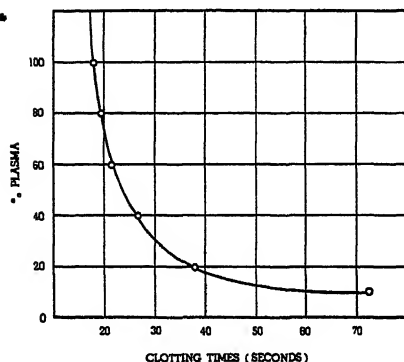


FIG. 1.

FIG. 1. Standard curve used in obtaining the figures given in parentheses in Table II

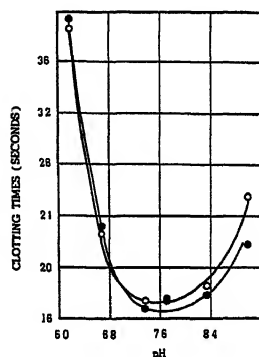


FIG. 2.

FIG. 2. The effect of pH on the clotting time of citrated human plasma as determined by Quick method

The Effect of pH on the Clotting Time. In order to study the effect of pH on the clotting time of plasma, samples of lyophilized plasma were restored with various dilutions of dilute (0.14 N) hydrochloric acid. The pH values of these samples ranged from 3.78 to 8.35. A sample restored with distilled water had a pH value of 8.96. The clotting times of the undiluted plasma samples were determined within 3 or 4 minutes of restoration by the method of Quick, using two different thromboplastin preparations. The data are presented in Fig. 2. At pH values of 3.78 and 5.0 (not included in the curve), no clot was observed in 2 minutes. It appears from these results that the optimum pH for the determination of the clotting time of citrated human plasma by the

Quick procedure lies between pH 7.4 and pH 7.8. The clotting times found at these pH values correspond very closely to the values found for the plasma prior to desiccation.

Numerous experiments not reported in this paper have demonstrated that the clotting times of restored plasma at a given pH value are the same within experimental error, regardless of whether the diluent used is dilute hydrochloric acid or carbon dioxide and water. Strumia's results (3) indicate that dilute citric acid (0.1 per cent) is a suitable diluent for the preparation of restored plasma that has a clotting time approximately equal to that of the original plasma. It is probable that the low prothrombin values found by Strumia when cold water saturated with carbon dioxide was used as a diluent were due to the presence of excess carbon dioxide in the diluting fluid, with a resulting low pH of the restored plasma.

Destruction of Prothrombin in Plasma Maintained at Various Levels of pH. The experiments recorded above indicated that the lyophile technic does not *per se* cause an appreciable destruction of the prothrombin of human plasma. However, it seemed advisable to investigate the stability of prothrombin in previously lyophilized plasma restored to various alkaline pH values. For this purpose, samples of lyophilized plasma were restored with distilled water and with dilute hydrochloric acid solutions to pH values of 9.2, 8.9 and 8.3, respectively. A control sample was restored with distilled water and carbon dioxide to pH 7.2, which was the pH of the original unprocessed plasma. After determination of the clotting times and pH values, all the samples were allowed to age at room temperature. At suitable intervals, samples were adjusted to pH 7.2 with carbon dioxide, and the clotting times were determined. By means of standard curves (6), these clotting times were converted to "per cent original prothrombin." The results of this experiment are summarized in Fig. 3. Each point represents the average of the results obtained with three separate thromboplastin preparations.

These data indicate that no appreciable destruction of prothrombin occurred in 24 hours at pH 7.2. At higher pH values, a progressive destruction of prothrombin with time was observed. The rate of this destruction was rather low, as evidenced by the fact that 81 and 89 per cent of the original prothrombin still was present at pH values 9.2 and 8.9, respectively, after one hour. Results at these pH values are mentioned because they are representative of the values found when commercial lyophilized plasma is restored with sterile distilled water. These

data indicate that, under usual conditions, the restored lyophilized plasma administered to human patients contains only slightly less prothrombin than was present in the plasma prior to desiccation.

Tocantins (4) reported that the loss of prothrombin activity that occurred when plasma was aerated was not affected by oxygen, and could be restored by carbon dioxide. Quick (5), on the other hand, was unable to restore the prothrombin activity of human plasma aerated at 38°C. to normal with carbon dioxide, and ascribed the loss of activity to some type of oxidative process. In the experiments reported in this paper, in which the destruction of prothrombin at alkaline pH values was

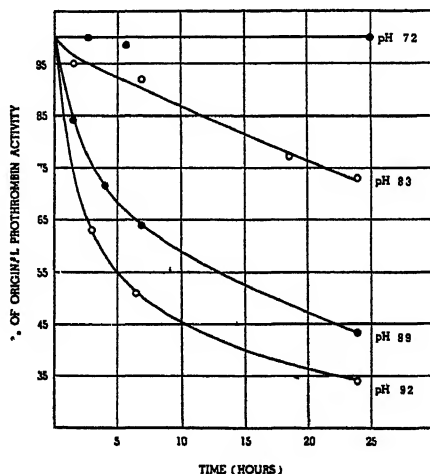


FIG. 3. Loss of prothrombin activity at various pH values

determined, the loss of activity took place in the presence of only minimal quantities of oxygen. The dried plasma used was contained in small vials under vacuum. When this plasma was restored with water or with dilute hydrochloric acid to provide solutions with pH values above physiological ranges, the vacuum was only partly displaced by the liquid. No air, except that dissolved in the diluent, was permitted to enter the vial until equilibration with carbon dioxide. The evidence presented in this communication does not support the hypothesis that the inactivation of prothrombin in alkaline plasma requires the participation of molecular oxygen.

EXPERIMENTAL DETAILS

Thromboplastin Suspensions. The thromboplastin suspensions used in this investigation were prepared from acetone- and dioxane-dried rabbit brain by minor modifications of the method described by Quick (7). In the case of thromboplastin No. 1 (see Table I), the suspension was centrifuged at 1800 r.p.m. for 10 minutes. The centrifugate was poured off, and the residue was extracted twice more with equal volumes of the oxalated saline solution. The three centrifugates were combined, placed in small vials, and lyophilized. In the preparation of thromboplastin No. 2, the suspension was centrifuged at 1800 r.p.m. for 10 minutes. After removal of the centrifugate, the residue was extracted once with a volume of oxalated saline solution equal to the volume of the original suspension, and the two centrifugates were combined, filled into small vials, and lyophilized. Thromboplastin No. 2 had been kept in the lyophilized state for approximately a year when this study was undertaken; thromboplastin No. 1 was a recent preparation. The other thromboplastin suspensions used were prepared by one of the methods outlined above. Prior to use, the thromboplastin preparations were restored to original volumes with distilled water.

Plasma. Plasma samples, approximately three days old, were obtained from pooled lots of 50 human bleedings. The anticoagulant used was sodium citrate (50 cc. of 4 per cent sodium citrate in 0.85 per cent sodium chloride solution for each 500 cc. of blood).

Equilibration with Carbon Dioxide. In some cases, equilibration with carbon dioxide was accomplished by restoring the lyophilized plasma with a diluent prepared by bubbling carbon dioxide through distilled water at 37–38°C. for five minutes. In other cases, the desiccated plasma was restored as follows: One cc. of distilled water was injected through the rubber stopper of the vial, which had a capacity of 2 cc., and which contained the equivalent of 1 cc. of fresh plasma. This procedure was carried out in such a way that a partial vacuum was maintained in the vial. One-half cc. of carbon dioxide gas was then injected through the stopper with the aid of a syringe and needle, after which the interior of the vial was brought to equilibrium with atmospheric pressure by the introduction of air. The restored plasma was allowed to remain in the stoppered vial (with occasional shaking) for five minutes before the stopper was removed.

pH Measurements. All pH determinations were made with a glass electrode assembly (Beckmann pH Meter).

SUMMARY

1. The lyophile technic does not cause an appreciable loss of the prothrombin activity of citrated human plasma.

2. The prothrombin level of lyophilized citrated human plasma remains essentially unchanged after storage at 5°C. for at least 45 days. Preliminary unpublished data suggest that no appreciable loss of prothrombin will occur after storage at various temperatures for long periods of time.

3. pH has a marked effect on the clotting time of human plasma, as determined by the Quick procedure. The optimum pH for prothrombin activity appears to lie between 7.4 and 7.8.

4. The pH of lyophilized citrated human plasma restored with distilled water varies between 8.8 and 9.3. The prothrombin level of this alkaline plasma slowly decreases with time. At pH values of 8.9 and 9.2, 89 and 81 per cent, respectively, of the original prothrombin activity still remain after storage of the restored plasma at room temperature for one hour.

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(See also discussion at the end of this reference)
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The Effect of Parenteral Injection of Synthetic Amino Acids upon the Appearance, Growth and Disappearance of the Emge Sarcoma in Rats¹

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During the past 12 years the writer has been studying the effect of administration of an aqueous extract of the cortex of the suprarenal gland of sheep and cattle upon the growth and regression of transplantable tumors in rats and spontaneous tumors in mice (1, 2). From this extract 7 amino acids were isolated and identified in 1934 (3). At this time it was evident that the type and proportion of these amino acids present in proteose and peptone form in the extract were important factors in causing about 10 per cent of the transplantable tumors to disappear. At this time, however, many of the synthetic amino acids were not available so that further studies along this line had to be discontinued.

At the present time a great deal of interest is being shown in the study of the effect of diet (proteins, amino acids, nitrogenous bases from muscle tissue, etc.) upon the growth and regression of experimental tumors in animals. For instance Creech and Franks (4) coupled the carcinogenic hydrocarbons with casein. The introduction of the carcinogenic hydrocarbon in one flank of the animal produced a tumor, while the introduction of the casein-carcinogenic hydrocarbon complex in the other flank produced no tumor. The casein completely prevented the tumor formation. Murphy (5) showed that a substance, protein in nature, exists in chickens tumors which is capable of preventing their

¹ Presented before the Chemical Symposium at the meeting of the American Association for the Advancement of Science, Dallas, Texas, December 29, 1941 and before the American Association for Cancer Research, Boston, Mass., March 31, 1942.

growth and which also neutralizes the transmitting factor in the tumors. The work of Hammett (6) showing that some of the amino acids promote regeneration and differentiation in *Obelia* is very interesting in this connection. The same is true of the work of Roffo (7) who showed that a neutralized hydrolysate of muscle tissue (upon which a tumor seldom develops in man) caused the complete disappearance of the growing tumors in rats in 3 weeks.

A personal communication from Professor Roffo states:

"The tumor regression obtained in these animals which gave me some hope at first did not take place in human tumors nor in rats with spontaneous malignancies. I think, therefore, that the muscle hydrolysate and its derivatives do influence the biological ground producing a biochemical modification. It is a satisfaction to me to learn from your letter that you also thought of the action of the amino acids, which is in conformity with some of my experiments performed more than a year ago. Concerning this point I will gladly give you some details which you may add to your communication on the subject: The proteins and polypeptides tested in rats did not produce any difference in tumor growth, while all of the amino acids and other substances tested (*D*-glutamic acid, *DL*-proline, *L*-leucine, *L*-histidine-HCl, glycyl-*L*-leucine, glycyl-*DL*-leucine, *DL*-alanyl-glycine, xanthine, hypoxanthine, guanine, and creatine), caused the complete disappearance of the tumors."

Boyland (8) has recently confirmed these results of Roffo. Administration of muscle extract and aliphatic bases caused the disappearance of tumors in rats. The active material was precipitated by phosphotungstic acid and was due to several bases rather than to any single one. Lustig and Wachtel (9) stated that guanidine acetic acid and creatine dissolved cancer cells *in vitro* and that guanidine derivatives prolonged the lives of tumor bearing animals. In some of our more recent studies arginine and histidine caused the disappearance of 8 tumors.

The above results show the importance of studying the effect of administering the pure, synthetic amino acids, alone and together, on the growth and disappearance of experimental tumors in animals. The results of our first study in this connection are listed below.

EXPERIMENTAL

The tumor used in these studies was obtained from Doctor Perry of the University of California who in turn secured it from Doctor G. L. Laqueur of the Department of Obstetrics and Gynecology of Stanford University. A personal communication from Doctor Laqueur stated:

"As to the tumor you have received, it is named the Emge-I-2 sarcoma in this laboratory (10). Its history is quite interesting because it developed from a spontaneous mammary adenofibroma into the present sarcoma through mere transplantation. The tumor is now in its 159th transplantation with practically 100 per cent takes in both males and females in our colony. To our knowledge and experience, the tumor never metastasizes but kills the host within 40 to 50 days after transplantation. It was, therefore, of greatest interest to me to learn from your letter that you had obtained tumors weighing more than 200 g. This is considerably more than we usually see without erosion of the skin." A further personal communication from Doctor Emge stated: "In many thousands of tumor transplantations which we have done in the past 14 years we have never encountered a spontaneous disappearance of a fully developed tumor. However, in hybrids developed from our pure strain by crossing with wild rats, we have observed the resorption of the very early growth phases."

Double transplants were made into opposite flanks of the abdomen of young rats, using the trochar method. Estimated tumor weights were made weekly by palpation of the small tumors in the abdomen, usually within about 10 per cent of their true weight. All tumors were removed and weighed at the death of the animal or at the end of the study. The amino acids were secured from Merck and Company, Rahway, N. J. and from the Department of Chemistry of the University of Illinois at Urbana.

All young rats were secured from the Albino Farms of Red Bank, N. J. They were fed *ad libitum* on Purina Dog Checkers. No record of individual food intakes was kept but it can be stated that all animals consumed adequate quantities of this food and made excellent gains in body weights. Animals weighing over 300 g. in perfect health at the end of 3 years have often been produced. The ingredients of the checkers were: meat meal, dried skim milk, wheat germ, barley malt, dried beet pulp, corn grits (from corn and wheat), dried raisins, soy bean oil meal, molasses, riboflavin concentrate, carotene, dried brewers' yeast, cod liver oil, 1 per cent each of steamed bone meal and iodized salt. Analysis in per cent: protein, 21; fat, 4; crude fiber, 6; and nitrogen-free extract, 46.

The composition of 6 liters of the amino acid solution in g. was as follows: *D*-arginine-HCl, 15; *D*-histidine-HCl, 3.6; *D*-glutamic acid, 15; *DL*-phenylalanine, 6; *DL*-alanine, 18; *L*-leucine, 6; *DL*-valine, 14; *L*-cystine, 15; *L*-proline, 3; *L*-hydroxyproline, 3. The first 7 of these were present in these proportions in the adrenal cortical extract (3) and the last 3 were added in the belief that they would be beneficial. One half quantities of the amino acids were weighed out and placed in a 4 liter beaker con-

taining 2.5 l. of sterile distilled water. They were just dissolved with the least amount of 10 per cent NaOH followed by neutralization to a pH of 7 by the addition of dilute HCl. The whole was then made up to 3 liters and kept in the ice chest when not in use. Some of the cystine precipitated out under these conditions so that the stated amount of this amino acid was not always administered. Three cc. of the mixture were injected daily except Sunday around the tumor or at the other sites in the abdomen. Injection of sterile physiological salt solution did not have any effect on the general characteristics of the tumors.

RESULTS

The average results obtained in Groups I to IX inclusive are listed in Table 1.

Control Group I. At the end of the study 44 out of 62 rats were living in this group. The total tumor weight was 1493 g., average 38 g., and the tumor incidence was 98 per cent. No tumor disappeared. In 12 other control groups in 7 studies with the Walker and Emge sarcomas in San Francisco and New Orleans, the average weekly growth rate of the tumors in g. from 3 to 7 weeks after transplantation was: 2.2, 5.1, 14.1, 25.2, and 33.1. No tumor in any of these groups disappeared in a total of 498 rats.

Exp. Group II. The amino acid solution was injected for 3 weeks beginning the day of transplantation of the tumors. At the end of the study 48 out of 70 rats had 19 tumors weighing 157 g., average 8 g., as compared to an average weight of 38 g. in Control Group I above. *Thirty-eight out of 46 tumors disappeared.*

Exp. Group III. The amino acid solution was injected beginning the second week after transplantation of the tumors for 2 weeks. For an additional week the amino acid solution was consumed by mouth in place of the drinking water. Fifty-five out of 102 rats were living at the end of the study and the 34 tumors weighed 478 g., average 14 g., as compared to 38 g. in Control Group I. The tumor incidence was 62 per cent. As far as the average growth of the tumors is concerned it is seen, by a comparison of the average growth rate of the tumors in Groups II and III, that it is better to begin injections of the amino acid solution the day of transplants rather than to wait two weeks during which the tumors have begun to grow. *Eighty-three out of 120 tumors disappeared in this group.*

Exp. Group III a. Double retransplants of tumor tissue were made

TABLE 1

Group	Weeks after transplantation of tumors														Remarks		
	2		7		2		7		2		7		2			7	
	Rats		Total tumor weight*		Average tumor weight		Tumors		Tumors		Tumors		Tumors disappeared				
	No.	g.	g.	g.	g.	g.	No.	No.	per cent	per cent	No.	No.	No.	No.			
Control I	62	44	47	1.493	0.8	38.2	61	39	98.4	88	6	0	0	Amino acid solution injected for 3 weeks after transplantation of tumors. Amino acids injected 2 weeks after transplantation of tumors for 2 weeks, then by mouth for another week.			
Exp. II	70	48	29	157	1.0	8.3	46	19	65.7	39	6	0	38				
Exp. III	102	55	139	478	1	2.14	0	120	34	117	6	61	8		0	88	
Exp. III a	57	56	64	100	5.3	33	0	12	3	21	0	5	4	0	Growth of retransplants in rats in whom the first tumors had disappeared. Growth of second spontaneous tumors in whom first tumors had disappeared. No retransplants.		
Control III b	60	58	54	265	4.2	44.1	13	6	21.7	10	3	0	7	0			
Control IV	19	13	18	420	1.8	26	3	10	16	52.6	123	0	1	40 cc. daily of the amino acid solution by mouth beginning the day of transplantation of tumors.			
Exp. V	14	14	4	50	1.0	10.0	4	5	28.6	35.7	0	0	5		0		
Control VI	28	25	18	1.464	1	0	52	3	17	28	60	7	112	0	Old rats. Old rats: 47 cc. daily of the amino acid solution by mouth first 3 weeks after transplantation of tumors.		
Exp. VII	28	26	15	610	1	0	32.1	15	19	53.4	73	0	9	0			
Control VIII	30	23	31	300	2.4	25	0	13	12	43	0	52	0	0	Same as Exp. group II above.		
Exp. IX	32	32	16	175	1.2	16	0	13	11	40	0	34	0	15			

* Total tumor weight at 2 weeks was estimated by palpation of small tumor in the abdomen; total tumor weight at 7 weeks was obtained by removing tumor and weighing at death of animal or end of study.

into the 57 rats of this group in whom first tumors had disappeared under amino acid administration. Two weeks later the tumor incidence was 21 per cent and at the end of the study it was 5 per cent. Out of 12 new tumors, 9 disappeared without any amino acid administration, leaving 3 at the end of the study. The tumor incidence in Exp. Groups II and III, from which these animals were taken, was 66 and 117 per cent, respectively. These results show that administration of the amino acid solution produces a lasting protection to second transplants of the tumors in rats. It was also observed that many tumors were held stationary in weight for as long as 4 weeks after the administration of the amino acid solution was discontinued, after which the tumors then began to grow at the same rate as the controls.

Control Group III b. This group served as a control for Exp. Group III a above. In 60 rats in whom first tumors had disappeared, 13 or 22 per cent of them with a total weight of 54 g. again developed second tumors spontaneously. Of these, 7 disappeared a second time without any further amino acid therapy, while the remaining 6 grew to a total of 265 g. This is further evidence of the protection conferred on the animals by the amino acid solution against the growth of some of these second tumors.

Control Group IV. One tumor disappeared in this group. The average increase in tumor weight was from 2 to 26 g.

Exp. Group V. These rats consumed an average of 40 cc. daily of the amino acid solution by mouth for 3 weeks beginning the day of transplants of the tumors. The average increase in tumor weight was from 1 to 10 g., as compared to 2 to 26 g. in Control Group IV above. Five tumors completely disappeared in Group V.

Control Group VI. Transplants were made into healthy 2 year old rats. The average increase in tumor weight was from 1 to 52 g. and no tumor disappeared.

Exp. Group VII. In another similar group of 2 year old rats the amino acid solution was ingested by mouth for the first 3 weeks after transplantation of the tumors. The average increase in tumor weight was from 1 to 32 g. and 9 tumors disappeared.

Control Group VIII. The average tumor growth was from 2.4 to 25 g. and no tumor disappeared.

Exp. Group IX. This is a repetition of the studies of Group II above. The average tumor growth was from 1.2 to 16 g. and a total of 15 out of 26 tumors, or 60 per cent, disappeared.

Exp. Group X. (Table 2.) Fifty-three rats of Control Groups I and IV above had 67 tumors weighing from 25 to 100 g. 6 weeks after transplantation of the tumors. Five to 10 cc. of the amino acid solution, depending on whether one or two tumors were present, were injected daily except Sunday around the tumors. Nineteen of the rats died the first day of the study before the amino acid solution was injected. Some of the animals of this group lived from 4 to 6 weeks longer than they would have otherwise. The amino acid solution evidently prolongs the life of the tumor bearing animals by relieving their cachexia and loss of body weight. Eight of these tumors, weighing from 25 to 100 g., completely disappeared, 2 liquified and 7 ulcerated. Fifteen weeks after

TABLE 2

Group	Weeks after beginning injections of amino acids																			
	0		5		0		5		0		5		0		5		0		10	
	Rats		Total tumor weight		Average tumor weight		Tumors disappeared		Tumors liquified		Tumors ulcerated		Living animals							
	No.	No.	g.	g.	g.	g.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
Exp. X.	53	16	2,038	433	67	11	0	8	0	2	0	7	0	9						

transplantation of the tumors 9 of these rats were living in perfect health.

DISCUSSION

Sufficient experimental data have been accumulated by ourselves and others to justify a few theoretical considerations. These are:

First, it is well known that cancer proteins are synthesized much faster than are normal tissue proteins. As the latter are converted into cancer proteins the tumor increases in size and the animal loses weight and dies from cachexia. It would seem that the animal cannot ingest sufficient protein to supply enough amino acids for the synthesis of the normal and cancer proteins at the same time. Or, more specifically, the growing tumor may greatly utilize a given amino acid, nitrogenous base, or group of these which are necessary for its own growth and this would result in a lack of these same substances for the synthesis of the normal tissue proteins.

By administering an extra supply of the amino acids we were interested

to find that many of the tumors disappeared and the animals seem remarkably healthy. Especially is this true if the tumors weighed less than 10 g. From the results published by Roffo (7), Boyland (8) and Lustig and Wachtel (9) it would also seem that nitrogenous bases, such as occur in muscle tissue, can also accomplish the same purpose. This is indeed interesting and we hope to attack this problem later.

Second, it is also possible that a growing tumor upsets normal protein synthesis in the area around it by more effectively utilizing enzymes necessary for this process. This view is analogous to the "conditioned deficiency" discussed by Rhoads (11) in regard to the carcinogenic action of *N,N*-dimethylaminoazobenzene (butter yellow). Kensler, *et al.* (12) have shown that this compound destroys some of the riboflavin of the liver and stops the growth of the animal. Feeding casein and riboflavin completely prevents the appearance of these tumors but does not cause them to disappear once they begin to grow. It is also possible that these normal enzymes needed for protein synthesis are destroyed by the growing tumor. Administration of extra amino acids might, therefore, provide raw material for the continued synthesis of these enzymes which would result in the continued growth of the animal and death of the tumor. We are, however, at a loss to understand why the tumor dies in this connection.

Third, it may also be that a certain type of antigen-antibody reaction occurs (Creech and Franks, 4). This is shown by the high degree of protection observed in many of our rats against a second transplantation of the tumors after the first tumors had disappeared under amino acid therapy, and to a lack of continued growth in new tumors which developed a second time on the original site in the animals.

Fourth, it is also possible that the amino acids may neutralize some agent which is essential for the growth of the tumor. This neutralizing action is occasionally seen in the prolongation of the life of animals bearing large tumors. Our results lend support to the current view of du Vigneaud, *et al.* (13) and Burk (14) that the protein avidin in raw egg white counteracts the growth promoting effect of biotin upon the tumors produced by feeding *N,N*-dimethylaminoazobenzene.

At the present time we have completed a study injecting each of the 21 amino acids of the protein molecule, alone, and also of the casein hydrolysate, Amigen, upon the growth and regression of these tumors. These results are very significant and will be published later.

SUMMARY

Purified, synthetic amino acids (*D*-arginine-HCl, *D*-histidine-HCl, *D*-glutamic acid, *DL*-phenylalanine, *DL*-alanine, *DL*-valine, *L*-leucine, *L*-cystine, *L*-proline, and *L*-hydroxyproline) were combined in a definite proportion and dissolved in dilute NaOH, neutralized with dilute HCl to a pH of 7 and administered to young rats bearing the Emge-I-2 sarcoma. The results obtained were as follows:

1. The tumor did not disappear spontaneously in any animal of the 12 control groups (498 rats) in previous studies in San Francisco and New Orleans. In the present study this was likewise true with 1 exception of 139 control rats. The weight of the control tumors in the present studies varied from 25 to 260 g.

2. Parenteral injection of 3 cc. of the amino acid solution daily around the tumor, beginning the *day of transplants*, caused 83 per cent (38 out of 46) in one group and 60 per cent (15 out of 26) of them in another to completely disappear.

3. Ingestion of the amino acid solution in place of the drinking water in another group, beginning 2 weeks *after* transplantation of the tumors, caused 69 per cent (83 out of 120) of them to completely disappear.

4. Growth of some of the tumors is held stationary for a period of 3 to 4 weeks after amino acid administration is discontinued. The tumors then begin to grow again.

5. Small tumors soften, become necrotic and disappear completely in 2 or 3 weeks; those weighing from 10 to 30 g. may first ulcerate and then break down and leave, in some cases, healthy tissue; those above 40 g. may also ulcerate and liquefy and in some cases break down, but death of the animal usually occurs from infection of the large denuded area or from other causes.

6. Retransplants in some of the animals of Groups II and III resulted in only 21 per cent takes, and 75 per cent of these tumors disappeared a second time without any amino acid therapy. The takes in this group on first double transplantation of the tumors were 66 to 117 per cent. It was concluded that the administration of the amino acids confers a lasting protection in some of the animals against the growth of new transplants.

7. In another 60 rats 21 per cent developed second tumors on identical sites where the first tumors had disappeared. Of these second tumors 7 disappeared without any amino acid administration.

8. In 28, 2 year old rats, 9 out of 19 tumors, or 47 per cent, disappeared after ingestion of an average of 47 cc. daily of the amino acid mixture for 3 weeks after transplantation of the tumors.

9. Injection of the amino acid solution into rats bearing large tumors prolonged the life of some of the animals and caused 8, or 11 per cent, of the 67 tumors weighing from 15 to 100 g. to completely disappear, while 2 liquefied and 7 ulcerated.

Four theories were suggested to explain the results obtained in relation to the disappearance^a of the tumors.

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The Effect of Administering Cyanamide, Guanidine Acetate and Sodium Benzoate, with and without Amino Acids, upon the Creatine Content of Rat Muscle

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On several occasions in our past studies on creatine formation in rats we have noticed that a toxic reaction in the animal usually causes a fall in the creatine content of its muscles (1). The toxicity in these cases was due to the injection of larger amounts of a given substance than the animal could tolerate. This raises the question as to whether creatine formation in the body might be due to a detoxication reaction involving the amino acids. The results of the present study show, however, that this is not the case. The study was, therefore, discontinued and a brief report on our findings will be made at this time.

EXPERIMENTAL

Young growing rats were fed a stock diet consisting of $\frac{2}{3}$ whole wheat flour, $\frac{1}{3}$ whole milk powder, together with 1 per cent of the weight of the wheat, each, as NaCl and CaCO₃. Different amounts of cyanamide or guanidine acetate were injected with and without similar doses of glycine and methionine. In another study sodium benzoate was fed with and without glycine. The muscle tissue of this group was fixed in Helly and Bouin fluids and stained with hematoxylin and eosin for histological study. Determinations of muscle creatine were made using the method of Rose, Helmer and Chanutin (2) employing the Fisher electrophometer for making color comparisons.

RESULTS

In Table 1 it is observed that injecting 10 to 100 mg. of cyanamide or similar amounts of guanidine acetate caused an average drop in muscle

creatine from 0.41 to 0.29, and from 0.41 to 0.28 per cent, respectively. These changes were independent of the size of the dose injected. The toxicity to guanidine acetate varies with the animal since 3 died on doses that did not effect others. From this table it is likewise seen that in-

TABLE 1

The Effect of Injection of Cyanamide and Guanidine Acetate, with and without Amino Acids, upon the Concentration of Muscle Creatine in the Rat

No.	Substance injected			Body wt.	Exp days	Muscle creatine		
	Methionine	Cyanamide	Glycine			Maximum	Minimum	Average
No.	mg.	mg.	mg.	g.	No.	per cent	per cent	per cent
Control								
40				80-170	1	0.46	0.38	0.41
13		10-100		70-150	1	0.37	0.19	0.30
10		10-100	10-100	53-140	1	0.36	0.15	0.26
14		10-100		102-161	1	0.36	0.18	0.27
10	10-100	10-100		116-270	1	0.39	0.29	0.32
		Guanidine acetate						
		mg.						
10		10-100		80-135	1	0.37	0.25	0.30
11		10-100	10-100	102-163	1	0.34	0.23	0.28
10		10-100		100-242	1	0.30	0.24	0.27
10	10-100	10-100		160-214	1	0.32	0.18	0.28
	Cyanamide	Glycine	Guanidine acetate					
	mg.	mg.	mg.					
6	10-60			70-126	2	0.39	0.28	0.36
6	10-60	10-60		95-120	2	0.45	0.37	0.40
6			10-60	78-117	2	0.44	0.38	0.42
6		10-60	10-60	110-149	2	0.44	0.36	0.39
Control								
15				135-160		0.47	0.37	0.42

jecting similar doses of glycine or methionine with cyanamide and guanidine acetate did not prevent the loss in muscle creatine that was obtained after injecting these latter substances alone. In other words the formation of creatine in the animal under these conditions was not due to a detoxication of these substances by the amino acids. It is also

seen that cyanamide and guanidine acetate likewise prevented the increased creatine formation that would have otherwise occurred from the injection of either of these amino acids.

Creatine formation in the body is a fairly rapid process. It was desired, however, to study these changes for a 2-day period (Table 1). At this time it is seen that there was little change in the concentration of muscle creatine with or without the injection of similar doses of the amino acids. The fall in muscle creatine at the end of 1 day is followed by a return to normal at 2 days. Cyanamide and guanidine acetate again prevented the increase in creatine formation that would have otherwise occurred from the injection of the amino acids alone.

TABLE 2

Effect of Feeding Sodium Benzoate, with and without Glycine, upon the Concentration of Muscle Creatine in the Rat

Rats	NaBz.	Glycine	Length of study	Muscle creatine		
				Maximum	Minimum	Average
No.	per cent	per cent	days	per cent	per cent	per cent
Control						
30			14-90	0.48	0.36	0.41
14	3		90	0.48	0.33	0.33
8	5		14	0.41	0.30	0.35
16	5	5	14	0.47	0.33	0.39

In another study sodium benzoate was fed with and without glycine. A concentration of 3 or 5 per cent of the stock diet as sodium benzoate for 90 and 14 days, respectively, caused only a slight drop in muscle creatine which was insignificant. The feeding of 5 per cent of sodium benzoate with a similar amount of glycine likewise caused little change in the average creatine content of the muscles. The feeding of 10 per cent sodium benzoate was very toxic since all animals died soon after ingesting the diet. It would again seem that feeding 5 per cent sodium benzoate does, however, prevent the increased creatine formation which would have otherwise occurred by feeding 5 per cent glycine (3).

White (4) studied the effect on growth when various substances were fed to benzoate-stunted animals. Glycine, sarcosine and glycolic acid caused a resumption of growth in these animals while other substances, including methionine and creatine, were ineffective in this connection.

SUMMARY

Studies were made to determine if creatine formation in the body from glycine and methionine was the result of a detoxication reaction. The injection of 10 to 100 mg. each of cyanamide and guanidine acetate, with or without similar doses of glycine or methionine, caused an average drop in muscle creatine from 0.41 to about 0.30 per cent. This lasts for one day only. The injection of cyanamide or guanidine acetate prevents the increase in creatine formation that would have otherwise occurred from the injection of the amino acids. The feeding of 3 or 5 per cent of the stock diet as sodium benzoate with or without 5 per cent glycine (in the last study), however, did not change the creatine content of the muscles nor change their histological structure. The feeding of 10 per cent sodium benzoate was very toxic to all animals.

It was concluded that creatine formation in the rat was not due to a detoxication reaction involving glycine or methionine.

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Preparation of Active Zymase Extracts from Top Yeast

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There are two methods available to obtain cell-free enzyme preparations from yeast, capable of fermenting sugar. The classical process is that of Buchner (1), who subjected mechanically ruptured fresh yeast cells to pressure (yeast press juice). The method which is more convenient and generally gives better yields than the press process, was introduced by the Russian scientists Iwanov and Lebedev. Iwanov (2) recognized in fundamental experiments by means of which the biochemical phosphorylation was discovered, that together with coagulable protein the zymase (called alcoholase) and the phosphorylase (called synthease) could be simply extracted with water from dried brewer's yeast (Hefanol) as well as from acetone bottom yeast (Zymin). These cell-free, colloidal solutions, which he called yeast-extracts, were capable of fermenting sugar in the presence of toluene. Iwanov states:

"Dissolution of the alcoholase can always be demonstrated and depends from the treatment of the yeast. Alcoholase is extracted from Hefanol; in the case of Zymin the alcoholase is mainly in the insoluble residue."

The extraction was made by maceration of one part dried brewer's yeast with five parts of water at room temperature, 12–20°C. This observation seems to have been forgotten. Lebedev (3) who some years later gave a precise statement of this behavior of brewer's yeast did not mention, as far as we can determine, Iwanov's observations; he called the cell-free extracts maceration juice and established the proportion of dried yeast to water, most favorable for the extraction, as 1:3 at 35°C.

By common experience brewer's yeast is used for the preparation of press juice as well as for extracts. In continental Europe brewer's yeast generally is bottom yeast, while in England brewer's yeast has top yeast properties. *Baker's yeast has not been found suitable for the preparation of cell-free juices*, except in a very few cases. Lange (4) obtained press juice from a fresh distillery-yeast which was,

according to his statement, capable of fermenting, but was considerably less active than press juice from bottom yeast. In six experiments with baker's yeast Wroblewski (5) only once found a press juice which did show a very weak fermenting power. Lebedev did not succeed in extracting an active juice from Paris baker's yeast. Neuberg and Czapski (6) first prepared a *juice from a dried top yeast* which was for some time available under the name of "Florylin"; in this extract, they established, at least one component of the zymase, the carboxylase, was active.

It seems to be Rinckleben (7), who first recommended disodium phosphate solutions (5%) for the extraction of zymase from yeast. In his experiments on plasmolysis of brewer's yeast he used Na_2HPO_4 in a 0.16 molar solution and obtained a juice, which was slightly active. After that Haeggund and Rosenquist (8) proposed to extract the dried top yeast with a 0.15 molar solution of disodium phosphate instead of water. In this way active zymase solutions could be obtained in some cases; Lipmann (9) recommended the same method of extraction with Na_2HPO_4 solution of the above mentioned concentration. Not every baker's yeast yields an active juice when extracted with this phosphate solution. This method failed in the case of a Bohemian press yeast and with a Dutch baker's yeast while it was successful with the Berlin distillery yeast "M".

We have found that a considerably active maceration juice can be obtained from four of the American commercially available baker's yeasts, i.e., Atlantic, Blue Ribbon, Fleischmann's, and National Grain. As it seems that K^- and NH_4^+ ions (10) are indispensable for some partial processes of the fermentation, we used for the extraction solutions of diammonium phosphate, dipotassium phosphate, or of ammonium sodium phosphate. Diammonium phosphate gave the best results. The phosphate ion itself is not essential, as positive results can also be obtained with solutions of borate, of glycocoll potassium, or of potassium bicarbonate, all at a pH of about 8.3. Diammonium phosphate is superior to all. Already during shaking of the dried yeast with the ammonium phosphate solution the pH declines from 8.3 to about 7.5 and is about 6.6 in the clear juice. The extraction can be done at 37°, at room temperature, or at +4°.

The dried American top yeasts show qualities which distinguish them in a remarkable way from the European strains. Their cell-free extracts exhibit an excellent fermentative power and contain a far greater amount of coagulable protein (2.6-3.3%), which may be the cause of their favorable properties. The protein content is three times that in German top yeast juices, where Neuberg and Czapski have found, after extraction (1:3) with water, 1.1%-1.75% only which after conversion into the relation 1:5 would be equivalent to 0.66%-1.05%. This high protein percentage possibly accounts also for the long preservation of

these extraction juices from the top yeasts. Kept in the refrigerator ($+4^{\circ}\text{C}.$) they generally show considerable fermentation even after 10 days. The coagulable protein, which disappears in European yeast juices, according to Goret and Hahn (11), usually after 4-5 days by action of the endotryptase, is still present after 10 days and then amounts to 95% of the original content under this condition.

It is not decided whether the diffusion of an active juice from dried yeasts should be attributed chiefly to osmotic action or whether enzymatic processes play a role, which alters the cell membranes. Sobotka (12) ascribes great significance to processes of the latter kind and mentions that yeasts, which possess the enzyme system necessary for release of the zymase, lose their zymase completely in the first extraction and make further extractions ineffective. This is not the case with our top yeasts, as we have seen, for instance, with Fleischmann's yeast and with National Grain yeast. After extraction with three parts of ammonium phosphate solution, a second extraction of dried Fleischmann's or National Grain yeast with half of this quantity gives a juice of considerable fermentative power and rich in protein, and even a third extraction may yield a juice which is active. That the dried yeast even after three extractions is not completely exhausted of zymase is demonstrated by the fact, that the solid residue still ferments glucose vigorously in the presence of toluene, and still more actively after addition of co-zymase. We used generally three or four times as much diammonium phosphate solution as dried yeast; in some cases five parts of ammonium phosphate solution can be applied for the extraction of one part of dried baker's yeast with satisfactory results.

We found Fleischmann's yeast and National Grain yeast very suitable for obtaining constant results, while the behavior of Federal yeast was inconsistent.

The dried baker's yeast was prepared (according to the directions given by Schroder in Munich and also by Lebedev for brewer's yeast) by drying the yeast which has been forced through a kitchen sieve for about seventy hours in porous paper boxes at room temperature; the layers should not be higher than 0.6 cm., or 0.4 cm. if the relative humidity of the air is high. During this process the yeast acquires a yellowish color. The dry granules thus obtained finally are ground by means of a hand mill and preserved in closed tin boxes.

The yeast juice which serves for so many biochemical purposes, is now easily available in good yield by following these directions and can be

prepared without difficulty in any laboratory, if necessary without an incubator.

Our thanks are due to the Fleischmann Yeast Corporation, to the Atlantic Yeast Corporation, to the Blue Ribbon Yeast Corporation, and to the National Grain Yeast Corporation which provided us with the yeasts needed for our experiments.

A typical *experiment* may be described as follows: 300 g. dried yeast (Fleischmann's or National Grain) are gradually added to a solution of 22 g. $(\text{NH}_4)_2\text{HPO}_4$ in 1000 cc. of tap water, shaking constantly to avoid the formation of lumps. The mixture which is placed in a bottle not too tightly closed because of auto-fermentation, is warmed in a water bath at 37°C . and then placed in an incubator at the same temperature for two hours with frequent shaking. Then the mixture is separated by means of a centrifuge or by filtering in the refrigerator through large filters. Centrifuging yields ca. 600 cc. of zymase solution. 20 cc. of this juice, after 1 cc. of toluene has been added, begin to ferment saccharose or glucose within a short time at 37°C . The induction period is reduced by addition of 0.5 cc. of an activator mixture of the following composition: 0.1 g. MnCl_2 , 0.1 g. MgCl_2 , 1.0 g. calcium-hexose-di-phosphate, 0.15 cc. acetaldehyde, and 1000 cc. of water. Manganese salt was added, since Kayser demonstrated its favorable effect on alcoholic fermentation, and, since he especially recommended the addition of traces of Mn^{II} for use with yeast press juice (13).

The extraction can also be carried out at room temperature (20° – 24°) instead of in the incubator at 37° . In this case the digestion is continued for five hours. This can be done also in the refrigerator at $+4^\circ$, as recommended many years ago for dried brewer's yeast (14); the extraction is then extended to 20 hours. The results obtained at different temperatures are not always identical. Quickly fermenting juices are also prepared by extraction with $\text{NH}_4\text{NaHPO}_4 \cdot 4\text{H}_2\text{O}$ (32 g.: 1000) under the same conditions. The extracts made with K_2HPO_4 (22 g.: 1000) generally take double the time before beginning to ferment; the extraction with an equimolar solution of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ (54 g.: 1000) gives inferior preparations.

Ineffective Na_2HPO_4 extracts can be made capable of fermenting. Neuberg and coworkers (15) demonstrated, that preparations of dried top yeasts, which were not phosphorylating could be induced to phosphorylate if an extract of boiled top or bottom yeast was added. This extract contains the co-enzymes, inorganic phosphates and ammonium

salts. As the phosphorylation of the hexoses is linked to fermentation, it could be expected, that addition of boiled yeast juice to ineffective zymase extracts would activate the latter. This is indeed the case. For instance, inactive extracts, made from National Grain yeast or from Federal yeast by extraction with Na_2HPO_4 solution, or juices, which had become inactive after preservation in the ice-box at $+4^\circ$ for some time, are reactivated, if 3-5% of ordinary boiled yeast juice is added, under otherwise equivalent conditions. The boiled yeast juice has been prepared from bottom or top yeast following the directions of Neuberg and Gottschalk (15). It can be kept in the refrigerator, with addition of toluene, for many weeks.

Protein-Content:

- (a) Juice, prepared by extraction of one part dried Fleischmann's yeast with five parts diammonium phosphate solution, contains 3.27% of protein (fresh).
- (b) Juice, prepared by extraction of one part dried Fleischmann's yeast with five parts dipotassium phosphate solution, contains 2.58% of protein (fresh).

After preservation in the ice-box at $+4^\circ$ for two weeks, the protein contents were in (a) 3.11% and in (b) 2.45%.

If the extracts are frozen (-6°) their efficacy is nearly undiminished after 10 weeks. Our results differ from those of Wieland (16) who observed a quick and irreversible inactivation of zymase solutions from brewer's yeast at extremely low temperatures. Nord and Franke (17) found that zymase solutions from bottom yeast do not change their fermentative power for two months. Our experiments demonstrate that frozen juices from top yeast also retain their ability to ferment (in some cases however only after addition of coferment). The originally clear juices show after repeated freezing and thawing the phenomenon described by Nord (18), i.e., becoming opaque until flocculation, caused by cryolysis.

SUMMARY

A dependable method for the formation of active zymase in cell-free yeast extracts has been available only with brewer's yeast. It has been shown that an active zymase solution can be obtained from different American commercial baker's yeasts by extraction with solutions of ammonium-salts, especially diammonium phosphate, in various simple ways. In this manner the zymase extract which is needed for many purposes can be prepared in any laboratory.

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Studies on the Biochemical Synthesis of Asparagine

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INTRODUCTION

The earlier work on the origin of asparagine in plants has been reviewed by Onslow (9), by Burkhart (3), and by Murneek (8), and was summarized briefly by Vickery and Pucher in a recent report (19). A comprehensive discussion of the whole problem of amide metabolism in plants is given by Chibnall (4), who presents in detail the evidence for the prevailing status of the problem.

Schulze's early views (14) still serve as a framework for the modern conception of the process of asparagine formation, namely, that ammonia from protein degradation products plus a nitrogen free organic acid derived from carbohydrate metabolism combine to form the amide. This postulate received support from the experiments of Suzuki (18), Prianischnikov (12), and Smirnov (16), and more recently from work by Mothes (7), Yemm (23), and Burkhart (3). Relative to the role of carbohydrates are data obtained by Petrie and Wood, which indicate that no correlation exists between carbohydrate content and asparagine formation, although ammonia appears to bear some relation to the amide content in the species studied by these workers (10).

The extensive studies of the immediate organic acid precursors of asparagine has yielded results which are difficult to compare and correlate, in part at least because of the wide variety of experimental techniques and plant materials employed. Complications also arise owing to the presence in the tissues studied of preformed protein and organic acids. An attempt to avoid these difficulties has been sought in the use of plant embryos, which contain only small amounts of such reserve material but which possess potentialities for growth and synthesis. Chemical changes occurring during growth on a known medium can

then be ascribed to synthetic anabolic reactions, which must ultimately depend upon the substrate as a source of supply of material.

MATERIALS AND METHODS

With the object of investigating a number of possible carbon skeleton precursors of the amide in the same *in vivo* system, experiments were carried out in an attempt to develop suitable methods for the *in vitro* cultivation of the excised seedling (radical, hypocotyl and epicotyl) of the lupin, a species known to synthesize significant amounts of asparagine. The method finally adopted was as follows: Lupin seeds (*Lupinus Hartwegii*, dark blue) were sterilized by a 2 minute treatment with 95 per cent ethanol, a 20 minute treatment with 0.1 per cent mercuric chloride, and a thorough washing in sterile redistilled water. The seeds were then germinated on moist filter paper in covered Petri dishes. After 3 days in the dark at 25°C., the seedlings were ready for culturing.

Using instruments dipped in alcohol and flamed, and working under glass in a steamed tile room sprayed with dilute Lysol solution, five plants at a time were transferred from the Petri dish to sterile filter paper. The two cotyledons were cut from each seedling and the latter "planted" in punctures in agar medium contained in 125-ml. cotton stoppered Erlenmeyer flasks. These flasks were then illuminated at alternate 12 hour intervals by an automatically controlled 100 watt Mazda lamp placed 18 inches above the flasks; the room temperature was 25°C. ($\pm 2^\circ$). The seedlings were taken for analysis after 12 days under the above conditions.

The basic agar medium contained per 100 ml. 0.1 mg. thiamin chloride, 3 g. sucrose (the sugar best utilized in these experiments), White's inorganic salts (22), 1.5 g. agar, and 0.3 mg. of each of the following amino acids:

<i>l</i> (-)-alanine	<i>dl</i> -leucine
<i>l</i> (+)-arginine	<i>l</i> (+)-ornithine
<i>l</i> (-)-asparagine	<i>dl</i> -phenylalanine
<i>l</i> (-)-cystine	<i>l</i> (-)-proline
<i>l</i> (+)-glutamic acid	<i>dl</i> -serine
glycine	<i>l</i> (+)-lysine-2HCl
<i>l</i> (-)-histidine-HCl	<i>l</i> (-)-tryptophane
<i>dl</i> -isoleucine	<i>l</i> (+)-valine

The latter compounds were chosen from those recommended for the growth of isolated roots (1, 22) and were added to supply trace amounts

of indispensable amino acids, rather than for the purpose of acting as mass nutrients. No attempt was made to work out specific requirements for amino acids. To the basic media were added substances or combinations whose ability to act as asparagine precursors it was desired to test. Each medium will be designated by this supplement only. The pH was adjusted to 7 with sodium hydroxide, using the glass electrode.

Following the 12 day culture period, from 20 to 25 plants were taken from each medium for analysis. After being carefully washed, the tissue was extracted with 4 per cent sodium sulfate as described in a previous paper (6), and the extract analyzed for the various nitrogen fractions by the micro-methods of Borsook and Dubnoff (2). To evaluate the order of magnitude of the deviations to be expected in the experimental results, certain of the experiments were completely replicated. The following data, which represents a composite of both sampling and analytical errors, are typical:

	Mean values per 50 seedlings	Probable error
Total N.	23.6 mg.	2.1
Protein N	3.9 mg.	1.3
Amide N	7.9 mg.	.3
Amino N	9.8 mg.	.4

RESULTS

Effect of the Ammonium Concentration. The classical explanation for asparagine formation in plants has been the so called "detoxication theory", which postulates that toxic ammonia arising from amino acid deamination is removed and stored as the innocuous amide. While there is ample evidence that ammonium fed to certain species via the substrate or through vacuum infiltration is partly converted to the amide, this process may not be exclusively a detoxication mechanism. *A priori* there is no more reason to consider ammonium as toxic than to view any other readily absorbed ion as toxic when supplied at high concentrations, and the very fact that ammonium can be utilized argues against such a view. A similar interpretation of the metabolism of ammonium ion has recently been expressed by Burkhart (3) and by Vickery and Pucher (19).

The effect of increasing ammonium concentrations on asparagine synthesis in isolated lupins is indicated in Table 1. Data on the seedlings at the end of the initial 3 day germination period are included to show

the order of magnitude of the synthetic processes occurring during the subsequent 12 day period of growth on the agar media.

Comparison of experiments 3 and 6 indicate that a forty-fold increase in the ammonium concentration of the medium increased the ammonium content of the tissues fifteen-fold but produced no corresponding increase in amide content. If one regards a positive protein balance as an index of the general synthetic ability of the tissue, there is no evidence of a toxic effect of relatively high ammonium concentrations. The slight effect of ammonium ion on amide formation lends support to the view that this substance is limiting to amide synthesis only at relatively low concentrations, as Vickery and Pucher found for tobacco leaves and

TABLE 1
*Nitrogen Distribution in the Lupin Seedling after 12 Days Growth
on Various Substrates*

Exp. no.	Medium	mg. N per 50 seedlings					Hypocotyl length
		Total	Protein	Amide	Amino	Am- monia	
							mm.
1	3 day old seedlings	8.1	3.8	0.8	2.6	0	10
2	Control. No $(\text{NH}_4)_2\text{SO}_4$	16.2	1.0	3.0	4.7	0	25
3	20 mg. % $(\text{NH}_4)_2\text{SO}_4$	21.6	4.5	6.5	8.1	.4	26
4	250 mg. % $(\text{NH}_4)_2\text{SO}_4$	26.9	4.0	6.9	6.9	3.0	27
5	500 mg. % $(\text{NH}_4)_2\text{SO}_4$	24.1	4.2	5.3	6.4	5.4	23
6	800 mg. % $(\text{NH}_4)_2\text{SO}_4$	27.0	4.4	6.0		6.6	27
7	1000 mg. % $(\text{NH}_4)_2\text{SO}_4$	24.0	3.6	4.7	5.5	6.7	23

rhubarb (19). At least in these three species the simple detoxication theory appears inadequate entirely to explain the experimental findings.

Four-Carbon Dicarboxylic Acids As Asparagine Precursors. On the basis of structure, certain four-carbon dicarboxylic acids are possible precursors of asparagine. Malic, succinic, and fumaric acids have been tested in various plants such as maize (16) and bean (7), but in many cases the results are difficult to interpret because the high initial protein and carbohydrate tend to obscure the origin of the amide subsequently arising. In repeating some of Mothes' experiments on the bean, Schwab claims to have shown that asparagine synthesis occurs independently of the organic acid supplied with the ammonium ion (15). In his experiments however, negative protein balances suggested derangement of anabolic reactions, with the formation of the amide possibly from

protein decomposition products when ammonium acetate, oxalate, and bicarbonate were infiltrated. Infiltration of ammonium malate and succinate permitted the maintenance of positive protein balances, thus suggesting effective utilization of these compounds in anabolic reactions.

TABLE 2
*Nitrogen Distribution in the Lupin Seedling after 12 Days Growth
on Various Substrates*

Exp. no.	Medium	mg N per 50 seedlings				Hypocotyl length
		Total	Protein	Amide	Amino	
4	Control with $(\text{NH}_4)_2\text{SO}_4$	26.9	4.0	6.9	6.9	27
8	250 mg. % malate	25.0	1.8	6.8	8.4	24
9	500 mg. % malate	14.8	2.7	3.9	5.5	27
10	100 mg. % fumarate	31.4	3.7	7.9	9.4	35
11	500 mg. % fumarate	18.0	2.3	5.6	6.6	18
12	500 mg. % succinate	21.3	4.4	5.5	6.6	21
13	500 mg. % maleate	37.2	3.2	9.4	11.0	26
14	570 mg. % aspartate, no $(\text{NH}_4)_2\text{SO}_4$	19.6	3.0	5.1	8.1	28
15	570 mg. % aspartate	30.6	7.1	8.1	10.2	22
16	250 mg. % asparaginate, no $(\text{NH}_4)_2\text{SO}_4$	41.0	9.3	10.3	13.8	26
17	250 mg. % asparaginate	47.0	8.4	13.7	19.3	30
18	630 mg. % glutamate, no $(\text{NH}_4)_2\text{SO}_4$	17.0	1.7	4.3	6.1	28
19	630 mg. % glutamate	43.0	6.2	13.3	16.0	25
20	50 mg. % glutamate	34.0	2.4	9.9	11.9	40
21	630 mg. % glutamate	19.1	2.8	4.3	6.2	21
	100 mg. % oxaloacetate, no $(\text{NH}_4)_2\text{SO}_4$					
22	100 mg. % oxaloacetate	28.5	3.6	8.4	10.8	19
23	630 mg. % ketoglutarate	18.5	2.9	5.5	6.2	14
24	315 mg. % glutamate	14.0	2.6	3.1	5.0	24
	285 mg. % aspartate, no $(\text{NH}_4)_2\text{SO}_4$					

The effects of some typical compounds on the formation of asparagine in lupins are shown in Table 2, experiments 8–13. The media contained 250 mg. % of ammonium sulfate unless designated otherwise, and thus experiment 4 may be regarded as the control. In the preparation of certain of these media it was necessary to autoclave the basic medium

separately from the ammonium salt and the supplement, in order to prevent partial breakdown of the sucrose.

While the experiments do not cover a wide range of concentrations, they indicate in a general way that none of the compounds tested, with the possible exception of maleic acid, exert a conspicuous effect on amide synthesis when added to the substrate. No explanation appears obvious for the effect of this latter compound.

Organic Nitrogen Compounds as Asparagine Precursors. It has been observed by many workers that organic nitrogen compounds are in certain cases able to exert a growth promoting effect on plants. Virtanen found that oats and barley grew well on substances diffusing from legume nodules attached to pea plants growing in the same container. Aspartic acid has been shown to arise as a synthetic product of nodule activity (21), and this compound supplied artificially acts as a good nutrient for legumes and barley, as do also glutamic acid and asparagine (5).

The logical precursor of asparagine is ammonium aspartate, which could be converted to the amide by loss of a water molecule. Experimentally, Smirnov (16), and Mothes (7) were unable to obtain evidence of such a transformation on supplying the compound to maize and beans. Using the technique already described several organic nitrogen compounds have been tested as asparagine precursors in the lupin seedling. The data are given in experiments 14-17.

Experiment 15 affords no convincing evidence for the absorption and conversion of aspartic acid to asparagine when the former is added to the substrate, due regard being given the controls. The experiments in which asparagine itself was supplied appear to confirm the postulate that the amide may be a precursor of protein under certain conditions.

The possibility exists that the 5-carbon homolog of aspartic acid, namely glutamic acid, could undergo oxidative deamination and decarboxylation to yield a reactive 4-carbon intermediate (4, Chapt. IX). This scheme is not supported by the experiments in which the common 4-carbon-acids were supplied, but the importance of glutamic acid in tissue respiration justified an investigation of its effect on asparagine synthesis. The data are presented in experiments 18-20. Glutamine amide nitrogen was determined in experiments 18, 19, and 20 by the method of Pucher, Vickery, and Leavenworth (13); the values in mg. of N per 50 seedlings were 0, 0.5, and 0 respectively.

These experiments afford evidence of a stimulating effect of glutamic acid on the nitrogen metabolism of the lupin seedling. Experiment 20 was carried out to determine whether the glutamate might be acting as a coenzyme in a nitrogen transferring system (transamination); if this were the role of the amino acid, wide differences in the amount supplied would cause only relatively small differences in the amount of amide synthesized. The results do not strongly support this possibility.

Data on experiments which attempt to clarify the action of glutamate are shown in experiments 21-24. The role of the amino acid does not appear to involve oxidative deamination to ketoglutarate with subsequent utilization (decarboxylation) of this compound, nor is the transfer of the amino group of glutamic acid to oxaloacetic acid indicated (Exp. 21). Such a transamination would be expected to yield aspartic acid to form asparagine. Oxaloacetate (100 mg. per cent) was about as effective as aspartate (570 mg. per cent). At concentrations significantly higher than 100 mg. per cent oxaloacetate was toxic.

DISCUSSION

The experimental technique employed in this study of compounds structurally related to asparagine has yielded essentially no evidence that these substances play an important role in the synthesis of the amide in the lupin seedling. Of a large number of organic nitrogenous compounds, including many not mentioned in the foregoing report, glutamic acid was found to have the greatest effect on the nitrogen uptake and formation of asparagine under the conditions outlined. Vickery, et al. (20) have observed that aspartic and glutamic acids are especially reactive in the nitrogen metabolism of the tobacco plant, Starc and Baumann (17) reported a "potentiating" action of glutamate on the respiration of pigeon muscle, and glutamic acid has been found to stimulate the growth of certain bacteria (11). It thus appears that this compound has important roles in animal tissues and bacteria as well as in plants, although its function in plant and bacterial cells is yet to be clearly elucidated.

It is desired to postulate here a possible role of glutamate, namely that of an energy supply. The standard free energy of synthesis of asparagine from ammonium aspartate at pH 7 is 3460 calories. Normally this energy requirement is probably furnished by coupled reactions of carbohydrate metabolism; in the presence of glutamate further

energy is made available from the intermediary metabolism of this compound. This interpretation emphasizes energy supply as a limiting factor in the synthesis, when nitrogen is present in adequate amounts.

SUMMARY

1. Ammonium ion added to the substrate was found to limit the formation of amide in the isolated lupin seedling at relatively low concentrations only.

2. Compounds structurally related to asparagine were in general found not to stimulate amide formation in the lupin seedling when added to the substrate. Aspartic and oxaloacetic acids in the presence of ammonium ion were included among the substances tested.

3. Glutamic acid in the presence of a suitable source of nitrogen stimulated the nitrogen uptake and synthesis of asparagine in the lupin seedling.

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Interference Between Inactivated Bacterial Virus and Active Virus of the Same Strain and of a Different Strain*

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INTRODUCTION

In the preceding paper (1) a case of interference between two bacterial viruses (Bacteriophages), α and γ , which are active on the same bacterial strain, has been described. Each bacterium infected with both viruses liberates only virus γ ; virus α is suppressed. One particle of virus γ is sufficient to suppress the growth of virus α in a bacterium. With mixed infection, the growth of virus γ on the bacteria is normal, unless virus α is present in very great excess. Only when virus γ reaches the bacteria several minutes later than virus α does the latter succeed in growing in some of the bacteria to the exclusion of the former.

The ability of virus γ to interfere with the growth of virus α represents a novel property of bacterial viruses. The present paper is concerned with attempts to dissociate the interfering from the reproducing capacity of the virus.

MATERIAL AND METHODS

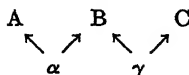
Most of the material and methods used in these experiments have been discussed in detail in the preceding paper (1).

The material consisted of two bacterial viruses, α and γ , both active on the same host, B (*Escherichia coli*). Each of them is active also on one of two bacterial

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† Fellow of the John Simon Guggenheim Memorial Foundation.

indicator strains, A and C, as shown in the following scheme, where arrows indicate susceptibility:



When either virus α or virus γ is grown separately, in the presence of bacteria of the strain B, the average yield of virus is 135–140 particles per infected bacterium, liberated at the moment of lysis. The minimum latent period is 13 minutes for virus α and 21 minutes for virus γ .

Methods. For the thermal inactivation, virus samples were put in a water bath of the desired temperature, withdrawn at intervals, and tested for the remaining virus activity.

For the inactivation with ultraviolet rays, the total radiation of a mercury lamp was used. The virus was exposed under standard conditions, and samples were removed from time to time. Absolute absorption doses (not given in this paper) would be of no significance, since, the virus being exposed in crude suspension, foreign material is largely responsible for the absorption.

For the growth experiments, the previously described "one-step growth" was used. Experiments for testing the interfering activity of variously treated samples of virus γ were generally made under standard conditions. Constant amounts of bacteria and virus α were mixed with the treated virus γ , and the growth of virus α was followed. Most experiments were run in parallel with a similar control: the sample of treated virus γ to be tested was replaced by the same amount either of broth, or of normal virus γ taken from the stock from which the treated sample came. Under standard experimental conditions, and in the absence of virus γ , the titer of virus α increases by a factor of 35–45 during the step of growth. The step begins 13 minutes and is completed 20–25 minutes after adding virus α to the bacterial culture. The reduction of this increase can be used as a measure of the amount of suppressing activity. Special experiments will be described in the next section.

EXPERIMENTAL RESULTS

A. Heat Inactivated Virus

Samples of virus γ , inactivated at a temperature of 55°C. until their titer (initially about 10^{10} particles/cc.) was reduced to 10^3 or less, were tested for interfering activity. The growth of virus α in the presence of heat inactivated virus γ , was always found to be normal. This shows that heat inactivated virus γ loses its interfering as well as its reproducing capacity.

B. Ultraviolet Inactivated Virus

I. Suppression of the Growth of Virus α by Ultraviolet Inactivated Virus γ

It was thought that inactivation by ultraviolet radiation would offer a favourable chance of conserving the interfering activity of the virus. It

is known that ultraviolet inactivated viruses are not so deeply altered that they lose their antigenic power, or their ability to act as vaccines (2, 3, 4).

The first experiments, with samples of virus γ completely inactivated by irradiation, showed immediately that this virus had maintained at least part of its ability to interfere with the growth of virus α . In a typical experiment, virus α increased six times, as compared with 35 times in the control experiment without virus γ . Controls with irradiated broth and bacterial filtrates never inhibited the growth of virus α . Following these preliminary results, different experiments were devised to study the dependence of the interfering activity on various factors.

TABLE I

The Growth of Virus α in the Presence of Virus γ Which Has Been Irradiated for Various Times

Experiment	Time of irradiation of virus γ	Residual titer of virus γ	Dilution of virus γ in the adsorption mixture	Increase of the titer of virus α
No.	minutes	virus/cc.		
53b	0	1.2×10^{10}	1.5/10	1.2 times
53a	5	3.5×10^4	1.5/10	2 "
64b	10	0	0.5/10	10 "
49	15	0	1.0/10	11 "
59	60	0	1.5/10	45 "
64c	Control (no virus)			40 "

1. *Different Irradiation Times.* Results are shown in Table I. The small amount of virus γ remaining in the less irradiated sample (5 minutes) is not sufficient to interfere with the growth experiments, as it can infect less than 1/10,000 of the bacteria.

It is seen that the suppression of the growth of virus α , which is practically complete with normal virus γ , is almost complete with virus γ irradiated five minutes; it diminishes progressively with irradiation times of 10 and 15 minutes, and is totally absent with virus irradiated for 60 minutes.

The virus α -suppressing activity of suspensions of virus γ is, therefore, progressively destroyed by increasing doses of ultraviolet rays. This activity is, however, much more resistant than the reproducing activity of the virus, since to destroy it requires much larger doses.

2. *Proof That the Inhibiting Action of Irradiated Suspensions of Virus γ Is Due to the Inactive Virus.* The interference between virus γ and virus α is strictly quantitative: every cell infected by one particle of virus γ becomes unable to reproduce virus α . Therefore, by increasing amounts of virus γ , more bacteria are infected, and virus α is more completely suppressed. It was desired to prove that the virus α -suppressing activity of irradiated virus γ suspensions followed the same quantitative relationship.

An experiment was performed, in which various amounts of irradiated virus γ were added to different portions of a mixture of bacteria and virus α , and the growth of virus α was followed.

Experiment No. 75. 1.0 cc. of a bacterial culture was introduced in each of three tubes. Tube 1 was inoculated with 0.01 cc. of inactivated virus γ (irradiated five minutes) + 0.005 cc. of virus α . Tube 2 was inoculated with 0.04 cc. of inactivated virus γ + 0.005 cc. of virus α . Tube 3 was inoculated with 0.1 cc. of inactivated virus γ + 0.005 cc. of virus α . After 4.5 minutes, the three mixtures were diluted and the growth of virus α followed.

Tube	Amount of irradiated virus γ	Increase of the titer of virus α
1	0.01 cc.	61 times
2	0.04 cc.	26 times
3	0.10 cc.	5 times

It is evident that the inhibition of the growth of virus α diminishes with the amount of the irradiated virus γ present. Although the experimental data do not permit a complete calculation, the amount of virus α growth in the three different tubes is that expected, according to the hypothesis that virus α does not grow in those bacteria which have adsorbed at least one particle of virus γ .

We conclude that the virus α -suppressing activity of irradiated suspensions is due to infection of the bacteria with the particles of inactivated virus γ ("partially inactivated particles").

3. *Proof That the Inactivated Virus Particles Are Adsorbed by the Bacteria.* The inhibition of virus α growth by inactivated virus γ offered a possibility of testing and measuring the adsorption of the partially inactive particles by the bacteria. If they are adsorbed by the bacterial cells, the interfering activity of a suspension should be reduced by "extracting" it with bacteria. The following experiment proves that the partially inactivated virus γ particles can be extracted with sensitive bacteria.

Experiment No. 64. 0.8 cc. of a bacterial culture was inoculated with 0.2 cc. of inactivated virus γ (irradiated ten minutes). The mixture was kept twenty minutes at 37°C., then centrifuged ten minutes. The supernatant was removed and tested for inhibiting activity upon virus α growth. Two controls were run: in one, the supernatant was replaced by broth, in the other by a corresponding amount of irradiated virus γ not previously extracted with bacteria.

Tube	Content	Increase of the titer of virus α
1	Bacteria + supernatant + virus α	32 times
2	Bacteria + irradiated virus γ + virus α	10 times
3	Bacteria + broth + virus α	38 times

The suspension extracted with bacteria shows very little interfering activity, while the control with irradiated virus γ not previously extracted with bacteria shows a definite inhibition of virus α growth. A rough calculation shows that the extraction has reduced the interfering activity to about one-tenth. This is in quantitative agreement with the known adsorption rate of virus γ by bacteria.

II. The Inhibition of Bacterial Growth by Ultraviolet Inactivated Virus γ

Although unable to reproduce itself on the sensitive bacteria, the partially inactivated virus γ might lyse the bacteria. This possibility was excluded by microscopic observation on agar of bacteria after treatment with inactive virus, which showed that these bacteria do not undergo lysis. However, the observation also showed that they do not divide, and remain unchanged for hours. Whether or not bacteria dividing at the moment of infection can carry this division step to completion, could not be decided, because microscopic observation always starts several minutes after bacteria and virus are mixed.

Suspensions of virus γ irradiated with very large doses of ultraviolet rays, enough to completely destroy their interfering activity, do not appear to inhibit the bacterial growth. This point, however, has been studied only qualitatively.

In order to study more closely the inhibition of bacterial growth, experiments were done, in which bacteria and partially inactivated virus γ were mixed, and the survival of bacteria studied by colony count. The number of colonies decreased progressively with increasing amounts of irradiated virus.

Table II shows an experiment in which various amounts of irradiated virus γ were mixed with equal amounts of bacteria. The mixtures were

diluted after five minutes, and the surviving bacteria counted by plating for colony count. The number of surviving bacteria diminishes as the amount of irradiated virus increases. If the growth of a bacterium is prevented by adsorption of one particle of virus, one should expect the fraction of surviving bacteria in the different mixtures to follow Poisson's formula:

$$\text{surviving bacteria} = e^{-n},$$

where n is the unknown number of virus particles adsorbed per bacterium (multiplicity of infection). The values of e^{-n} in the different mixtures are experimentally determined, and the values of n thus obtained should be proportional to the concentration of irradiated virus. In other words, the ratio of the values in Column 4 to those in Column

TABLE II

Survival of Bacteria after Treatment with Different Amounts of Irradiated Virus γ

Experiment No. 79. Different amounts of virus γ irradiated for five minutes were added to different portions of a bacterial suspension. After five minutes the mixtures were diluted, and dilutions plated for bacterial colony count.

Tube	Concentration of irradiated virus relative to Tube 1	Surviving bacteria	Multiplicity of infection (calculated by Poisson's formula)	Multiplicity Virus concentration
		<i>per cent</i>		
1	1	0.26	5.95	5.95
2	0.5	5	2.99	5.98
3	0.3	15	1.90	6.33

2 should be constant. This ratio is listed in Column 5. The result agrees with the theoretical expectation. We conclude, that a bacterium is inhibited from growing whenever it has adsorbed at least one partially inactive particle of virus γ . This experiment permitted us to calculate that our virus sample, after five minutes irradiation, contained 4×10^9 partially inactive particles of virus γ /cc. Since the original stock before irradiation contained 1.2×10^{10} particles of active virus/cc., we conclude that with such a dose of radiation about two-thirds of the virus have been completely destroyed.

III. The Interference of Partially Inactive Virus γ with Active Virus γ

In the preceding paper (1), it was shown that single or multiple infection of a bacterium with the same virus produces no difference in the final

yield of virus particles per bacterium. To explain this fact, the existence of "self-interference" was proposed. Accordingly, only one virus particle is able to reproduce itself in each bacterium. Both "self-interference" and " γ versus α interference" were tentatively explained as competitive blockade of a key-enzyme present in the cell in limited amount. If this hypothesis is correct, ultraviolet inactivated virus γ , which can still interfere with the growth of virus α , should also be able to interfere with the growth of normal virus γ .

To test this possibility, irradiated virus γ was put into the presence of bacteria at various intervals of time before or after the active virus γ . Table III shows the results.

The growth of active virus γ is strongly reduced in the presence of inactivated virus γ . This reduction is more evident when the active

TABLE III
Suppression of the Growth of Virus γ by Irradiated Virus γ

Experiment	Amount of irradiated virus γ	Interval between the introduction of irradiated virus γ and of normal virus γ	Amount of growth of virus γ in per cent of the growth occurring in the absence of irradiated virus γ
<i>No.</i>		<i>minutes</i>	<i>per cent</i>
73a	1.5/10	+4	20
72b	1.5/10	+1.5	35
73b	1.5/10	-2	>90
18	0		100

virus reaches the bacteria several minutes after the inactive, and conversely, it almost disappears when the active virus is given a few minutes precedence. These results prove that there is interference between inactive and active virus γ . The fact that the amount of interference depends on the order in which the two reach the bacteria strongly supports the idea of a mechanism of interference by blockade of a bacterial constituent.

IV. The Relation between the Inhibition, by Partially Inactivated Virus γ , of Bacterial Growth, α -Growth and γ -Growth

Ultraviolet inactivated virus γ has been shown above to interfere with the growth of the bacteria, and with the growth of both virus α and virus γ on the bacteria. To investigate the quantitative relationship

between these three actions, experiments were done in which the suppression of bacterial growth by inactive virus γ was compared under identical conditions with the inhibition of the growth of viruses α and γ .

Experiment No. 72. A bacterial culture, after being assayed for bacterial titer, was divided in three portions (Tubes 1, 2, 3). At time zero, to each tube 15 volume per cent of inactivated virus γ (irradiated five minutes) was added. After 1.5 minutes, 0.5 volume per cent of virus α was added to Tube 2, and 0.5 volume per cent of active virus γ was added to Tube 3. After 6.5 minutes, the three mixtures were diluted. Dilutions from Tube 1 were plated for bacterial count. The growth of virus α and of virus γ was followed in the dilutions from Tubes 2 and 3. The amount of growth of the viruses was compared with the growth expected in the absence of irradiated virus γ .

Tube	Content	Result
1	Bacteria + inactive virus γ	Bacterial count reduced to 0.85 per cent
2	Bacteria + inactive virus γ + virus α	Growth of virus α reduced to 0.75 per cent
3	Bacteria + inactive virus γ + virus γ	Growth of virus γ reduced to 35 per cent

The results of this experiment show that the inhibition of bacterial growth and of virus α growth are approximately the same, whereas the inhibition of γ growth is much less complete.

This difference cannot be due alone to the fact that some bacteria become infected with active virus γ earlier than with inactive virus γ . In the experiment number 73a (see Table III) the inactive virus could infect practically all cells before the introduction of the active virus; yet the latter grew in a relatively high percentage of the bacteria. It appears, then, that active virus γ can grow in some of the bacteria infected with ultraviolet inactivated virus γ , although these bacteria have lost the capacity, both of reproducing themselves and of allowing the growth of virus α .

V. Partially Inactive Virus Does not Reproduce in the Bacteria

It was possible that bacteria infected with ultraviolet inactivated virus γ might liberate virus γ in the partially inactive form, although they do not undergo lysis. In this case, the newly formed virus, being deprived of the capacity of lysing bacteria, would not be detected by the usual plaque counts. On the other hand, the interfering activity of the irradiated virus γ should be found to increase in the presence of bacteria.

Filtrates from suitable mixtures of bacteria and irradiated virus γ , and from their sub-cultures, were tested for interfering activity and were always found to lack it. Therefore, we conclude that irradiated virus cannot reproduce itself on the bacteria in the partially inactive form.

VI. *Ultraviolet Inactivated Virus α*

Suspensions of virus α , inactivated by ultraviolet rays, were found to exert no inhibiting activity, either on bacterial growth, or on the growth of either virus α or γ . It is, therefore, impossible even to decide whether this inactive virus is still adsorbed by the bacterial cells.

DISCUSSION

Suspensions of a bacterial virus γ , after inactivation by ultraviolet rays, may conserve the ability to interfere with the growth of another virus α acting on the same host. The interfering activity is present only in irradiated suspensions of virus γ , not in bacterial filtrates, or in irradiated suspensions of virus α . It is destroyed by heat together with the virus activity, and it is adsorbed by bacteria at the same rate as the active virus γ . Finally, the amount of virus α -suppressing activity is in such quantitative relation with the amount of inactive virus present as to prove that the adsorption of one inactive particle of virus γ on the bacterial cell is sufficient to inhibit the growth of virus α . We conclude that irradiated particles of virus γ , although they have lost their reproducing activity, may keep their ability to interfere with the growth of virus α (partially inactive particles).

The ultraviolet inactivated virus γ also shows the capacity of inhibiting the growth of the sensitive bacteria. These are not lysed, but deprived of the ability to divide. The number of bacterial cells thus affected corresponds to that of the cells in which the growth of the virus α is inhibited; the two actions evidently are manifestations of the same phenomenon. The suppression of bacterial growth must be due to inhibition by virus γ of some fundamental step in the synthetic processes of the bacterial life cycle. With normal virus γ , this inhibition is not observable because of the lysis of the cell; with the inactivated virus, which has lost the lysing capacity, the inhibition becomes apparent.

The growth of normal virus γ , also, can be inhibited by the previous action of ultraviolet inactivated γ on the cells. However, this suppression is not as extensive as the inhibition of the growth of virus α .

Altogether, the experiments described above lend support to the hypothesis (1) that the interference between viruses γ and α is due to competition for some material, probably of enzymatic nature, necessary for virus reproduction and present in limited amount in the cell. Virus γ , either normal or partially inactivated by ultraviolet radiation, is capable of combining with this material to the exclusion of virus α . Inactive virus γ can also, to a certain extent, exclude active virus γ from this material. The suppression of bacterial growth by partially inactive virus γ further suggests that the blocked enzyme is intimately connected with the mechanism of bacterial division.

The interesting experiments of Andrewes and Elford (5) on the "killing" of bacteria by virus in the presence of sodium citrate may be quoted in support of our interpretation of the bacteriostatic action of irradiated virus. These authors found that in the presence of citrate the growth of the bacteria is instantly inhibited by the addition of virus, although the virus does not grow and the cell is not lysed. The citrate, by precipitating the calcium ions, prevents virus growth but not virus adsorption. The analogy between this and our present case lies in this: in both cases virus is adsorbed but unable to grow and in both cases bacterial growth stops almost instantly.

The interfering ability of virus γ is more resistant to irradiation than is the reproducing property, since it is present when the latter is destroyed. Nevertheless, the interfering property, too, is progressively destroyed by increasing doses of irradiation.

The inactivation of bacterial viruses and of other viruses by ultraviolet and x-radiation has been proved to be of a direct type (6, 7, 8, 9, 10). It has been shown to result from the absorption of radiation within the virus particle. Therefore, the smaller the volume to be hit by radiation, the more resistant is the virus particle. The fact that the interfering activity of virus γ can stand larger doses of radiation than the reproducing capacity suggests that its suppression requires, either an extensive damage to the virus particle by multiple acts of absorption, or the destruction of a different part (enzymatic property?) of the particle.

Our results show the possibility of rendering bacterial cells insensitive to viruses by treatment with ultraviolet inactivated virus. An extension of this possibility to the field of plant and animal viruses brings us to consider the production of ultraviolet vaccines. Ultraviolet irradiated rabies virus has been successfully used by Hodes, Webster and Lavin (3) and by Webster and Casals (4) as anti-rabies vaccine. Jungeblut and Sanders (11) mention the fact that a murine strain of

poliomyelitis virus, after ultraviolet irradiation, may retain the ability to interfere with the normal monkey strain.

Results obtained with bacterial viruses may not be applicable in their entirety to the case of viruses acting on more complex hosts. They may bear more similarity to plant than to animal viruses, since immunity and interference, in plants as in bacteria, are known to be strictly cellular.

Our results would suggest that in order to be used as a cell protecting vaccine, a virus should receive the *minimum* dose of radiation sufficient to destroy its infectivity, because the protecting activity is itself slowly destroyed by the radiation. Since partially inactivated virus cannot reproduce itself, the vaccine should be used in amounts large enough to block most or all of the "spots" in which active virus could grow (sensitive cells or cell components).

Although the situation in the case of animal viruses is complicated by the occurrence of serological mechanisms of immunity (12), it is possibly not meaningless that the results obtained with ultraviolet irradiated anti-rabies vaccine (3, 4) seem to be in agreement with the above suggestions.

SUMMARY

1. It is shown that a bacterial virus, γ , after inactivation by ultraviolet radiation, retains its ability to interfere with the growth of another virus, α , acting upon the same host. A *single* partially inactivated particle is sufficient to suppress the growth of virus α in one bacterium.

2. The partially inactivated virus γ is adsorbed by the sensitive bacteria, and it inhibits their growth without producing lysis.

3. The partially inactivated virus γ interferes also with the growth of active virus γ .

4. The interfering activity of virus γ , although more resistant to radiation than the reproducing activity, is progressively destroyed by larger doses of ultraviolet rays.

5. These results are interpreted as supporting the hypothesis that interference between bacterial viruses is due to competition for a "key-enzyme" present in limited amount in each bacterial cell. They suggest that this enzyme is also essential for the bacterial growth.

6. The bearing of these results on the problem of anti-virus vaccines produced by irradiation is discussed.

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Vitamin Deficiencies of Twelve Fungi

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In recent papers from this laboratory we have reported on the vitamin deficiencies of a number of fungi (1, 2, 3, 4, 5). In the present paper the responses of additional organisms to thiamine, pyridoxine and biotin are recorded.

METHODS AND MATERIALS

The following fungi were kindly supplied by Dr. Ross W. Davidson:

Ceratostomella (*Grosmannia*) *leptographioides* Davidson. Isolated from heart wood.

Chalaropsis thielavioides. Isolated from elm roots.

Polyporus versicolor. No. 71700-R.

Pholiota adiposa. No. 72077-S.

Stereum murrayii. No. 71162.

Schizophyllum commun. No. 71982-R.

Dr. Donald E. Bliss generously furnished the following organisms:

Chalaropsis thielavioides B247 Peyronel. Received from Peyronel Jan. 1934. Isolated from *Lupinus albus*.

Chalaropsis thielavioides B655, Peyronel. Isolated Sept. 1940 from rose at San Jose, California, by K. F. Baker.

*Ceratostomella*¹ (*Thielaviopsis*) *paradoxa* (de Seynes) Dade. B-520, B-521 and B-520 x B-521. These cultures came indirectly from Dade.

In addition the following fungi were used:

Schlerotinia sp. No. 3-931 isolated from *Opuntia*. Received from Dr. H. H. Whetzel.

¹ Also called *Endoconidiophora paradoxa*.

Sclerotinia sp. No. S-965 isolated from egg plant. Received from Dr. H. H. Whetzel.

Claviceps purpurea. Isolated from rye in 1941. Received from the University of Minnesota.

Ceratostomella sp. From London plane tree received from Dr. J. M. Walker. This is a strain which produces perithecia.

The fungi listed above were grown in pure culture on agar slants in test-tubes, each containing approximately 8 ml. of medium. The basal medium was composed per liter of 50 g. dextrose, 1.5 g. KH_2PO_4 , 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g. asparagine and 1.5 per cent purified² agar, supplemented with the following trace elements in p.p.m. 0.005 B, 0.02 Cu, 0.1 Fe, 0.01 Ga, 0.01 Mn, 0.01 Mo, and 0.09 Zn.

Each fungus was grown on the basal agar medium and the same medium plus per tube 5 μM moles thiamine, 5 μM moles pyridoxine and 0.05 μg . biotin methyl ester, singly and in all possible combinations; and also on the basal agar medium plus 0.3 g. malt extract per tube. Stock cultures were maintained on the basal agar medium plus thiamine and peptone and inoculations of the agar slants were made by transferring a bit of mycelium about the size of a pin head. The cultures were incubated at 20°C. and observations on growth were made at frequent intervals.

Those fungi which showed vitamin deficiencies on the agar media were grown also in 125 ml. Erlenmeyer flasks each containing 25 ml. of the basal medium but without agar and in the same medium supplemented per flask with 10 μM moles thiamine, 50 μM moles pyridoxine and 0.1 μg . biotin methyl ester, singly and in all possible combinations; and also in the basal medium plus 0.3 g. malt extract per flask. Mycelium was used in inoculating the liquid cultures of *Schizophyllum commune*, *Polyporus versicolor*, *Stereum murrayii*, *Pholiota adiposa* and *Sclerotinia* sp. #S-931 and #S-965. A drop of a spore suspension in distilled water was used for the others. Dry weights of the growth in the liquid cultures were determined by washing the mycelium with distilled water and drying at 100°C.

All cultures were grown in triplicate. Glassware was cleaned with chromic-sulfuric acid cleaning mixture, rinsed in tap water and distilled water. Merck's synthetic thiamine and pyridoxine and S.M.A. crystalline biotin methyl ester

² Difco agar was purified by leaching with 5 per cent aqueous pyridine, followed by 1 per cent HCl. The agar was then saturated with $\text{Ca}(\text{OH})_2$ and the excess calcium washed out with distilled water.

were used. The asparagine was purified by treatment with Norit A and precipitation from alcohol. The dextrose was Corn Products C.P.

OBSERVATIONS

Ceratostomella leptographioides grew on the basal agar medium. Growth was improved by the addition of thiamine, of pyridoxine and of thiamine and pyridoxine, but was still better in those tubes which contained biotin, biotin and thiamine, biotin and pyridoxine, or all three vitamins. The growth on malt agar was not much better than that on agar containing the three vitamins. After 25 days incubation the mycelium had covered the agar slopes in those tubes containing vitamins while on the basal medium only about one half the surface was covered. *C. leptographioides* appeared to suffer from partial deficiencies, especially for biotin. As was observed earlier for some other fungi which suffer from partial deficiencies (4) this organism responded favorably on an agar medium to additions of thiamine, of pyridoxine, or of biotin.

C. leptographioides grew in the basal liquid medium (Table I) and neither thiamine nor pyridoxine materially affected growth. Biotin alone, or in combination with the other vitamins, was decidedly favorable. Sub-cultures from each kind of solution were made into similar solutions. The fungus grew in sub-culture in the basal solution evidencing its lack of any complete vitamin deficiency. In the sub-cultures in the liquid media, biotin favorably influenced growth, and neither thiamine nor pyridoxine appeared to exert any beneficial effect. The dry weights for the growth in the thiamine solution in both passages do not agree with those found in other solutions. We have no explanation for these apparent irregularities.

Ceratostomella (Thielaviopsis) paradoxa. The two sexual strains (*Thielaviopsis*, B-520 and B-521) of *C. paradoxa* and the mating (B-520 x B-521) showed very much the same responses on the agar media. Growth was sparse even after 40 days on the basal medium, and on that supplemented with pyridoxine, with biotin, or with biotin and pyridoxine. Heavy growth developed on the media supplemented with thiamine, with thiamine and pyridoxine, with thiamine and biotin, and with all three vitamins. Within six days the slopes of the tubes containing thiamine alone, or in combination with the other vitamins, were covered with a thick cottony mycelium (Fig. 1). However, growth on the malt agar was considerably better than on any of the media supplemented

with vitamins. Both sexual strains when grown alone or combined, suffered from a complete thiamine deficiency. Perithecia were observed on the malt agar of B-520 x B-521 when examined after 42 days incubation, but not elsewhere.

One strain only, B-521, was grown in liquid culture. The results confirmed those found on the agar medium; the fungus showed a complete deficiency for thiamine, and a marked response to malt extract.

Chalaropsis thielavioides. The three isolations of *C. thielavioides* were grown on the agar media. The responses of all of them to the three vitamins were much the same. After 42 days incubation very little growth had developed on the basal medium and on that supplemented with pyridoxine, with biotin, or with pyridoxine and biotin. Growth was heavy on the media containing thiamine, thiamine and pyridoxine, thiamine and biotin, or all three vitamins. Development on the malt agar was not quite as rapid or as good as on the agar containing vitamins. *C. thielavioides* suffered from a complete thiamine deficiency.

The results in liquid culture confirmed those obtained with the agar media (Table I).

Ceratostomella radiculicola grew extremely little on the basal medium, and on the same medium supplemented with pyridoxine, with biotin, or with pyridoxine and biotin. A very scanty growth developed in those tubes containing thiamine, and a little more in those containing thiamine and pyridoxine. Growth on the media supplemented with thiamine and biotin, or with all three vitamins, was rapid and heavy. Within six days the agar slopes were covered with a bluish grey or thick black mycelium (Fig. 1). The growth on malt agar was somewhat heavier than on the media supplemented with vitamins. This fungus appeared to suffer from a complete deficiency for thiamine and nearly complete deficiency for biotin.

The growth in liquid cultures confirmed these conclusions. There was a slight response to thiamine; a heavy one to thiamine and biotin (Table I).

Ceratostomella from *London Plane Tree*. This perithecia-producing strain showed a complete thiamine deficiency, and a partial deficiency for pyridoxine (Fig. 1). After 42 days, growth on the basal medium and on that supplemented with pyridoxine, with biotin, or with biotin and pyridoxine, was so small as to be almost invisible. On the media supplemented with thiamine, or with thiamine and biotin, the colonies were about 35 mm. in diameter while the entire surface of the slopes was

covered with mycelium in those tubes containing thiamine and pyridoxine, all three vitamins or malt. *Perithecia* were freely produced on all media containing thiamine alone or in combination with the other vitamins. This fungus was not grown in liquid culture.

TABLE I

Average Dry Weight Produced in a Mineral Dextrose Solution Containing Asparagine and Supplemented as Indicated

Ceratostomella radicicola, *Endoconidiophora paradoxa* and *Polyporus versicolor* grown 11 days; *Schizophyllum commune* and *Ceratostomella leptographioides*, 12 days; *Chalaropsis thielavioides*, 26 days; *Pholiota adiposa* and *Sclerotinia* No. 931, 47 days.

Additions to 25 ml. of basal liquid medium	<i>Ceratostomella radicicola</i>	<i>Endoconidiophora paradoxa</i>	<i>Polyporus versicolor</i>	<i>Ceratostomella leptographioides</i>	<i>Ceratostomella leptographioides</i> (sub-cultures)	<i>Schizophyllum commune</i>	<i>Chalaropsis thielavioides</i> C117	<i>Chalaropsis thielavioides</i> B 247	<i>Chalaropsis thielavioides</i> B 655	<i>Pholiota adiposa</i>	<i>Sclerotinia</i> sp. No. S 931
None.....	3.0	1.2	0.7	30.3	79.3	2.7	0.3	0.6	0.2	4.8	32.8
10 mμ moles thiamine.	13.1	119.0	24.2	60.5	36.7	35.0	33.2	28.1	54.8	31.5	144.2
50 mμ moles pyridoxine.....	2.7	1.1	7.9	31.8	81.0	2.3	0.5	0.2	0.2	11.7	46.2
0.1 μg. biotin.....	2.6	2.0	8.5	124.8	104.2	5.6	1.6	0.1	1.6	10.1	41.3
Thiamine and pyridoxine.....	1.9	119.6	54.2	33.9	62.5	31.4	35.7	43.8	106.5	41.7	114.4
Thiamine and biotin	201.0	123.3	24.7	127.1	103.9	41.6	41.5	55.1	89.9	49.4	122.8
Pyridoxine and biotin.....	2.5	1.1	8.1	109.7	110.2	2.7	0.4	0.3	0.4	9.1	22.9
Thiamine pyridoxine and biotin.....	231.5	127.6	47.6	123.5	104.0	56.2	31.6	70.4	60.2	46.7	140.1
0.3 g. malt extract....	241.5	327.0	82.2	136.3	107.4	214.0	23.5	14.8	24.0	112.7	352.5

Claviceps purpurea grew on the basal agar medium and showed little response to thiamine, pyridoxine or biotin alone or in combination. Growth on malt agar was somewhat more rapid than on the media containing the vitamins.

Sclerotinia sp. No. S-931 showed a partial thiamine deficiency on the agar media. It grew slowly on the basal medium, and on that supplemented with pyridoxine, with biotin, or with pyridoxine and biotin.



FIG. 1. Three species of *Ceratostomella* grown on a mineral-dextrose medium containing asparagine and purified agar plus (1) nothing; (2) thiamine, (3) pyridoxine; (4) biotin; (5) thiamine and pyridoxine; (6) thiamine and biotin; (7) pyridoxine and biotin, (8) the three vitamins (A) *Ceratostomella paradoxa*; (B) *Ceratostomella* from London plane tree; (C) *C. radiculicola*. All cultures 24 days old.

After 42 days incubation the colonies on these media were 20 mm. in diameter while in those tubes containing thiamine, thiamine and pyridoxine, thiamine and biotin, or all three vitamins, the agar slopes were

covered with mycelium. Growth on malt agar was more rapid than on the agar containing vitamins.

The growth in liquid culture supports the statement that this *Sclerotinia* suffered from a partial thiamine deficiency. It grew in the basal solution, but more rapidly in the presence of thiamine.

Sclerotinia sp. No. S-965 grew rapidly on the basal medium covering the surface of the agar slopes within six days. Addition of thiamine, pyridoxine or biotin, alone or in combination, had no visible effect on the growth. Development on the malt agar was heavier than on the agar media containing vitamins.

Polyporus versicolor grew slowly on the basal agar medium and on that supplemented with biotin. After 30 days incubation the growth was very scant on these media. It was a little heavier on the media supplemented with pyridoxine, or with pyridoxine and biotin. The agar slopes in the tubes containing thiamine, thiamine and pyridoxine, thiamine and biotin, or all three vitamins, were covered with mycelium within a period of 18 days. Growth on malt agar was decidedly better than on the media containing vitamins. This fungus appeared to suffer from a nearly complete thiamine deficiency and a partial pyridoxine deficiency.

These results were in general confirmed by the liquid cultures (Table II). Growth was materially improved by thiamine; but with thiamine and pyridoxine the dry weight was twice that obtained with thiamine, or with thiamine and biotin. Growth in the liquid containing malt was nearly twice that obtained in any of the solutions supplemented with vitamins.

Schizophyllum commune grew slowly on the basal agar medium and on that supplemented with pyridoxine, with biotin, or with pyridoxine and biotin. On all the media containing thiamine growth was much more rapid. It was still better on the malt agar. After 30 days incubation colonies on all the media lacking thiamine were from 30 to 40 mm. in diameter with thin and sparse mycelium; on those containing thiamine a thick cottony mycelium covering the entire slope had developed. This organism appeared to suffer from a partial thiamine deficiency.

This conclusion was supported by the results with the liquid cultures. The liquid cultures also showed the marked favorable effect of a medium containing malt as compared to that containing vitamins; nearly four times as much dry matter developed in the liquid cultures containing malt extract as in any of the solutions supplemented with vitamins

(Table I). Sub-cultures of *S. commune* were grown from each of the solutions given in Table I. The dry weights of the second passage showed no material difference from those in the first passage.

Pholiota adiposa grew on the basal agar medium. Pyridoxine and biotin appeared to have little effect. Thiamine improved growth somewhat, but malt agar was superior to any of the media containing the

TABLE II
Vitamin Deficiencies of 12 Fungi and Response to Malt Extract

Fungus	Deficiency for		Biotin	Response to malt
	Thiamine	Pyridoxine		
<i>Ceratostomella leptographioides</i>	Partial?	Partial?	Partial	Little
<i>Ceratostomella</i> (<i>Thielaviopsis</i>) <i>paradoxa</i> , both sexes and the mating	Complete	None	None	Marked
<i>Ceratostomella radiculicola</i> .	Complete	None	Nearly complete	Little
<i>Ceratostomella</i> , from London plane tree perithecia-producing strain... ..	Complete	Partial	None	Medium
<i>Chalaropsis thielavioides</i> , three strains..... .	Complete	Slight or none	Slight or none	None
<i>Claviceps purpurea</i>	None	None	None	Medium
<i>Sclerotinia</i> sp., No. S-931.....	Partial	None	None	Medium
<i>Sclerotinia</i> sp., No. S-965.....	None	None	None	Medium
<i>Polyporus versicolor</i>	Nearly complete	Partial	None	Marked
<i>Schizophyllum commune</i>	Partial	Slight or none	Slight or none	Marked
<i>Pholiota adiposa</i>	Partial	Slight or none	Slight or none	Marked
<i>Stereum murrayii</i>	None	None	None	Marked

vitamins. This organism appeared to suffer from a minor partial deficiency for thiamine. A sporophore was observed in one tube of malt agar after 130 days, but none was noted in any of the tubes supplemented with vitamins.

P. adiposa grew slowly on the basal liquid medium. The addition of pyridoxine, or of biotin, improved growth somewhat but thiamine was considerably more effective. Over twice as much growth was

obtained in the liquid medium containing malt extract as in any of those supplemented with vitamins.

Stereum murrayii grew slowly on the basal agar medium; after 30 days incubation colonies 9 or 10 mm. in diameter had developed. Addition of thiamine, pyridoxine or biotin, singly or in combination, did not affect the growth. The development on malt agar was several times that on any of the media containing vitamins though the slopes were not entirely covered with mycelium even after 30 days incubation.

DISCUSSION

Nine of the twelve fungi included in this study suffered from complete or partial deficiencies for thiamine, pyridoxine or biotin. For five of them the deficiency was complete, or nearly complete, with the result that little or no growth occurred in a medium limited to minerals, dextrose and asparagine. Nine of the fungi showed deficiencies of various degrees for thiamine; three for pyridoxine, and two for biotin. The three species (*Claviceps purpurea*, *Sclerotinia* sp. No. S-965 and *Stereum murrayii*) which did not respond to thiamine, pyridoxine or biotin grew considerably better in a medium supplemented with malt extract than in that containing the three vitamins. This suggests that they have deficiencies for other growth substances. Some of the other organisms, notably *Endoconidiophora paradoxa*, *Polyporus versicolor*, *Schizophyllum commune* and *Pholiota adiposa*, also grew much better in the malt medium than in that supplemented with the three vitamins. Although this may be the result of some other cause than growth-substance deficiencies the obvious explanation is that thiamine, pyridoxine and biotin do not supply all the essential metabolites for which these organisms are partially or completely deficient. This is supported by the observation that *Pholiota adiposa* formed sporophores and *Endoconidiophora paradoxa* produced perithecia only on the malt agar. We should expect these structures to be produced on the vitamin media also if those media were entirely adequate.

A partial thiamine deficiency for *Schizophyllum commune* was reported by Robbins and Kavanagh (2), and by Schopfer and Blumer (6). Schopfer and Blumer (7) found this fungus to respond to the pyrimidine portion of the thiamine molecule.

Schopfer and Blumer (7) found *Polystictus (Polyporus) versicolor* grew poorly in a mineral-dextrose solution containing asparagine. Addition of thiamine increased growth fifty times. A mixture of the thiazole

and pyrimidine intermediates of thiamine was nearly as effective as thiamine, but either alone was ineffective.

The perithecial strain of the *Ceratostomella* from the London plane tree did not differ materially in response to thiamine, pyridoxine and biotin from that of the non-perithecial strain previously investigated (4).

Ceratostomella (Thielaviopsis) paradoxa obtained from Bliss showed essentially the same vitamin deficiencies as those evidenced by a strain obtained from Davidson (5).

Ceratostomella leptographioides on the agar media appeared to respond to thiamine, to pyridoxine or to biotin (in the liquid medium thiamine and pyridoxine were not effective). This sort of response was observed previously with some other fungi which showed partial deficiencies. We do not believe, however, that this is evidence for the assumption that one of these vitamins can replace another, but assume that where partial deficiencies exist an adequate supply of one vitamin may make it possible for the organism to synthesize larger quantities of the others.

Three isolations of *Chalaropsis thielavioides* were grown on agar and in liquid media. Although the three differed somewhat in their appearance on agar, their vitamin deficiencies were essentially similar. We have grown eleven isolations of *Ceratostomella montium*, kindly supplied by Dr. Caroline Rumbold, on the basal agar medium supplemented with thiamine, pyridoxine and biotin, singly and in all combinations. These were collected in different regions of the Rocky Mountains and from five species of pine trees. They were associated with two species of the bark beetle *Dendroctonus*. Although these isolations differed somewhat in rapidity of growth and appearance of colonies they all showed essentially the same vitamin deficiencies as reported earlier for this species (4). It is not surprising that the physiological characteristics of a species as well as its morphology should be relatively constant.

We did not determine the synthesis of vitamins by the fungi included in this investigation. However, previous observations (4) on other fungi showed that each fungus synthesizes from the constituents of the basal medium the vitamins for which it is not deficient. For example, it was found that *Ceratostomella ulmi*, which suffered from a pyridoxine deficiency, synthesized thiamine and biotin when grown in a solution containing pyridoxine. On evidence of this character we assume the beneficial effect of thiamine, pyridoxine or biotin on the organisms included in this study to result from their inability to construct sufficient of the particular vitamin. Conversely, where the vitamin has no bene-

ficial influence it may be assumed that the fungus synthesizes from the constituents of the basal medium sufficient of the particular vitamin.

This report is in the nature of a preliminary survey which raises many questions unanswerable without more detailed and exhaustive study of individual organisms. It is hoped that this may be undertaken, and that some of the fungi we have found to evidence vitamin deficiencies may be found useful in the study of that important group of substances.

SUMMARY

Twelve species of fungi were grown in a basal mineral-dextrose medium containing asparagine and supplemented with thiamine, pyridoxine and biotin singly and in all possible combinations. Nine showed partial or complete deficiencies for one or more of the vitamins. Three showed no response to the three vitamins, but grew better in a malt medium than in the basal medium.

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The Pigment of *Mimulus longiflorus* and the Isolation of its γ -Carotene Component

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As far as we know the pigment of the orange "Monkey flowers" (*Mimulus longiflorus*, Grant, Scrophulariaceae) has not been described. The present investigation was induced by the fact that chromatograms of the flower extracts showed the presence of substantial amounts of γ -carotene, $C_{40}H_{56}$. This carotene was detected by Kuhn and Brockmann (1) in commercial carotene where it occurs in quantities of about 0.1%. Neither this occurrence nor any natural source reported up to the present time (2-8), with the exception of the marsh dodder investigated by Mackinney (9), constitutes an easily available starting material for γ -carotene.

The pigment of the *Mimulus* flowers, which grow wild in Southern California, shows considerable variation in its composition. While the pigment of one of our materials contained 60 mg. of lycopene, $C_{40}H_{56}$, per kg. of dry flowers and a nearly equal amount of zeaxanthin, $C_{40}H_{56}O_2$, another was free of zeaxanthin but contained cryptoxanthin, $C_{40}H_{56}O$, and an increased quantity of lycopene (95 mg.). The photometrically estimated γ -carotene contents were 45 mg. and 75 mg. per kg. respectively. In our best experiment 45.5 mg. of crystals per kg. were isolated, i.e., about 60% of the γ -carotene content. The total amount available was 280 mg. of crystals.

No "pro"-carotenoids, which contain several *cis* double bonds (10), were found in Monkey flowers which were grown and fully opened under natural conditions. One of the authors (S.) observed (11), however, that Monkey flowers grown under certain conditions contained considerable amounts of pro- γ -carotene, $C_{40}H_{56}$, and polycopene, $C_{40}H_{56}$,

* Contribution No. 910.

instead of the corresponding all-*trans* carotenoids. The two pro-carotenoids were present in flowers which developed when stems with buds were placed in water in the diffuse light of the laboratory for several days. It is possible, therefore, that pro- γ -carotene and prolycopene are precursors of the ordinary forms.

The γ -carotene isolated from Monkey flowers has been found to be identical with samples isolated in this laboratory from other sources in all but one of its properties (including non-separation in a mixed chromatogram). A considerable difference exists, however, in the melting points of various samples (Table I). In spite of repeated purifications by

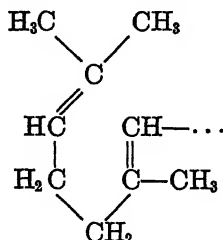
TABLE I
Melting Points of γ -Carotene Samples Isolated from Various Sources

Source	Plant organ	M p.° (cor)	Authors
Commercial carotene (Hoffmann-La Roche, Basel) ..		178	Kuhn and Brockmann (1)
Commercial carotene (Barnett Labs., Long Beach)....		151	(Authors, unpublished)
<i>Rosa rugosa</i>	Fruit	176.5	Willstaedt (5)
<i>Gonocaryum pyriforme</i>	Fruit	172	Winterstein (2, 3)
		160-165	
<i>Convallaria majalis</i>	Fruit	170	Winterstein and Ehrenberg (4)
<i>Cuscuta salina</i> }	Stem	164-165	Mackinney (9)
<i>C. subinclusa</i> }			
<i>Cuscuta californica</i>	Stem	131	(Authors, unpublished)
<i>Daucus carota</i>	Root	146	(Authors, unpublished)
<i>Gazania rigens</i>	Petals	131-133	(Authors, unpublished)
<i>Mimulus longiflorus</i>	Flowers	150	(This paper)

several methods including the full procedure described by Kuhn and Brockmann (1), it was not possible to raise the melting point above 150° which is 28° below the highest published. Although the crystals, purified according to these authors, melted at 150°, they melted originally rather sharply at 133°. This melting point was observed (within a degree or two) for all once recrystallized samples obtained in the various isolations. The analytical data, chromatograms of crystals, and microscopic investigation pointed to purity. The melting point, 133°, was not changed by simple recrystallization from different solvents, but only by the Kuhn and Brockmann (1) method. After the melting point, 150°, had been thus attained, change of solvent did not alter it. Our γ -carotene isolated from commercial carotene showed a similar behavior.

Table I demonstrates that the melting point data for γ -carotene vary much more than is common in the field of the carotenoids.

Because of the differences observed it was desirable to determine whether the isolated double bond is in the same position in our compound as in the γ -carotene of Kuhn and Brockmann (1) which contains one isopropylidene group. If this double bond were shifted to form an isobutylidene group, the maxima in the visible spectrum would not be affected since the double bond would not come into conjugation with



Aliphatic end-group of γ -carotene

the main chromophore. However, one might expect that the melting points of the two compounds would be different. A micro-determination of the isopropylidene group in the *Mimulus* γ -carotene according to the method of Kuhn and Roth (12) gave very nearly the theoretical quantity of acetone calculated for one isopropylidene group. The correctness of the figures obtained has been checked by micro-isopropylidene determinations with related compounds as listed in Table II. The structure of the aliphatic molecule end of γ -carotene from *Mimulus* and that isolated by Kuhn and Brockmann (1) is, therefore, the same.

The cause of the melting point variation cannot be given with certainty. If it does not result from a purely crystallographic phenomenon, the presence of small amounts of a stereoisomer with very similar adsorption affinity should be taken into consideration.

It has been mentioned (13) that the γ -carotene zone of some plant extracts seemed to consist of two components in the Tswett column. This was frequently observed on the first chromatogram of the crude extracts. The heterogeneity usually did not appear on a second chromatogram. It was not observed in *Mimulus* extracts in the present investigation although it had been previously (in 1941).

The *cis-trans*-isomerization of γ -carotene under various conditions will be described later.

Acknowledgment. We are indebted to Dr. G. Oppenheimer and Mr.

G. Swinehart for microanalyses and wish to thank Miss E. Estes, Mr. W. Scott, and Mr. I. Fatt for technical help.

EXPERIMENTAL

Materials and Methods. Calcium hydroxide was used as the adsorbent (Shell Brand lime, chemical hydrate, 98% through 325 mesh). The petroleum ether had a boiling range of 60–70°.

The concentration of the pigment solutions was determined with a Pulfrich Gradation Photometer (Zeiss, light filter S47). The photometric values for lycopene and β -carotene were those given by Chohnoky (14). γ -Carotene was calculated as $\frac{1}{2}$ (β -carotene + lycopene). Spectral maxima were taken with an Evaluating Grating Spectroscope (Zeiss, light filter BG-7) in petroleum ether unless otherwise indicated.

The melting points are corrected and were determined in a Berl block heated electrically to give a temperature rise of 2–3° per minute. The samples were sealed in tubes filled with carbon dioxide and were placed into the block 20° below the melting point.

The flowers were collected in Southern California during June and dried at 45–50° for 24 hours. (The dry weight was 23–24%.) The material was then kept under carbon dioxide in the dark and worked up within two weeks. A total of about 10 kg. of dry material was available, corresponding to 400,000–500,000 flowers.

Composition of the Pigment. For the quantitative determination of the individual carotenoids, the procedure previous to chromatography was that described below for large scale experiments. Twenty grams of dried and milled flowers were used. The following chromatogram was obtained on a column (20 × 3 cm.) after developing with petroleum ether containing 5% acetone (on the left the width of the zones is given in millimeters):

- 11 yellow and red minor top zones
- 1 colorless interzone
- 12 red: lycopene (506, 474.5, 446 m μ .)
- 13 orange: neolycopene (497.5, 465.5, 436.5 m μ .)
- 3 colorless interzone
- 20 orange: cryptoxanthin (484.5, 453.5 m μ .)
- 7 yellow: unidentified (471.5, 442.5 m μ .)
- 10 colorless interzone
- 45 pink: γ -carotene (495, 462, 433 m μ .)
- 27 pale orange: partially neo- γ -carotene
- 7 colorless interzone
- 25 to the bottom and filtrate: β -carotene (485, 452.5 m μ .)

The photometrically estimated content of the γ -carotene zone was 75 mg. per kg. of dry flowers and was less than the lycopene content (95 mg.). In flowers collected in 1941, however, only 45 mg. of γ -carotene per kg. was found while the lycopene value was 60 mg.

Isolation of γ -Carotene. 3.3 kg. of dried and milled Monkey flowers were extracted with petroleum ether in a large percolator ($45 \times 20 \times 8$ cm.). The material was moistened and covered with solvent. After standing overnight, the dark red-brown extract was drawn through by suction. As the top of the material became relatively dry, fresh solvent was continuously added. The total volume of the extract was 5 liters, the last portion of which was only faintly colored. These operations required 7 hours.

The extract was kept in large, well filled Erlenmeyer flasks over concd. methanolic potassium hydroxide for a day. After cautious addition of water, the petroleum ether phase was washed alkali-free. No carotenoids could be extracted from the dark aqueous layer. The deep red petroleum ether solution was dried with sodium sulfate, halved and chromatographed simultaneously in two percolators ($35 \times 15 \times 8$ cm.). Each chromatogram was developed with 1 liter of petroleum ether containing 2.5% acetone and then with 2 liters containing 5% acetone. In the course of the development β -carotene and substances which fluoresced in ultraviolet light were washed into the filtrate. The colored layers which appeared were the same as those indicated above. After the last portion of the developer had been drawn into the column, the percolator was inverted and the cone removed by tapping the glass. The irregularly shaped γ -carotene zone was cut out as well as possible and eluted with alcohol. After the adsorbent had been removed by filtration through a sintered glass funnel, the pigment was transferred into petroleum ether with water. It was washed alcohol-free, dried with sodium sulfate and developed on two cylindrical columns (28×7 cm.) with petroleum ether containing 5% acetone. After separating from the minor zones above and below it, the γ -carotene was eluted with peroxide-free ether.

The solution was evaporated to dryness in an all glass apparatus *in vacuo* under carbon dioxide. The dark red, glassy residue crystallized on cooling. It was dissolved in the minimum amount of benzene and transferred into a 50 cc. centrifuge tube with a dropper. Upon careful addition of excess methanol while stirring, red needles of γ -carotene

crystallized out.¹ After standing for 15 minutes, the crystals were centrifuged, washed with methanol, partially dried with a stream of carbon dioxide, and recrystallized from benzene-methanol as above. (Petroleum ether + methanol or carbon disulfide + abs. ethanol are also suitable crystallizing mixtures.) The suspension of γ -carotene was kept in ice water for an hour.

Macroscopically, the crystals appeared almost purple. The microscope showed very small clustered crystals. They tended to be rhombic in shape. Each rhombus was orange brown but regions where two were superimposed appeared intensely orange or reddish orange. After filtering and drying *in vacuo*, the yield was 150 mg. or 45.5 mg. per kg. of dry flowers. Experiments starting with 1 kg. and 2 kg. yielded 18 mg. and 30 mg. of crystalline γ -carotene per kg. respectively. The combined mother liquors from all three experiments gave 9 mg. The total quantity of γ -carotene available was 280 mg. The crystals were chromatographically homogeneous when a solution was washed down to the bottom of a very long tube (47×1.3 cm.).

The compound was epiphasic when partitioned between petroleum ether and 90–95% methanol.

Absorption maxima were as follows: in carbon disulfide, 532.5, 494.5, 461 $m\mu$.; in benzene, 509.5, 476, 447 $m\mu$.; and in light petroleum, 495, 462, 433 $m\mu$. Upon addition of iodine the maxima shifted to 529.5, 491.5, 458 $m\mu$.; 505, 472.5 $m\mu$.; and 491.5, 459 $m\mu$. respectively.

For analytical purposes the samples were dried in high vacuum at 56° for 40 min. They were free of ash.

Analysis.—2.371 mg. subs.: 7.764 mg. CO_2 , 2.283 mg. H_2O . — 2.391 mg. subs.: 7.878 mg. CO_2 , 2.250 mg. H_2O .

$\text{C}_{40}\text{H}_{56}$. Calculated. C 89.48, H 10.52

Found. C 89.36, 89.91, H 10.78, 10.53

Mol. weight. 0.314 mg. in 2.123 mg. of exaltone ($k = 21.3$): $\Delta = 6.4^\circ$

$\text{C}_{40}\text{H}_{56}$. Calculated. M. W. 537. Found. M. W. 492

Estimation of Isopropylidene Groups. Upon ozonolysis 16.93 mg. and 22.12 mg. of γ -carotene gave acetone corresponding to 3.76 cc. and 4.82 cc. of N/20 iodine respectively. After subtracting the blank value of 0.23 cc., the volumes were 3.53 cc. and 4.59 cc.

¹ The suspension of crystals should not be cooled because this causes a precipitation of colorless material. If such a complication should occur, the contaminant can be removed from the γ -carotene by centrifuging, adding methanol and warming in a water bath until the methanol boils for several minutes. After rapidly centrifuging, the hot methanol is decanted from the γ -carotene crystals.

Calculated. $(\text{CH}_3)_2\text{C}=\text{}$ groups per mole 1.0

Found. $(\text{CH}_3)_2\text{C}=\text{}$ groups per mole 0.95, 0.95

Control determinations are given in Table II.

Other Pigments. In one experiment 20 mg. each of zeaxanthin and lycopene per kg. of dry material were isolated in crystalline form. These carotenoids, as well as cryptoxanthin and β -carotene (which were not crystallized) were identified by mixed chromatograms, by spectra and by chromatographic positions. The absorption spectra in light petroleum were: zeaxanthin, 484.5, 453.5 $\text{m}\mu$.; lycopene, 506, 474.5, 446 $\text{m}\mu$.; cryptoxanthin, 484.5, 453.5 $\text{m}\mu$.; and β -carotene, 485, 452.5 $\text{m}\mu$.

TABLE II

Determination of Isopropylidene Groups in Some Carotenoids

(The values have been corrected for the blank)

Compounds	$(\text{CH}_3)_2\text{C}=\text{}$ groups per mole	
	Calculated	Found*
α -Carotene, $\text{C}_{40}\text{H}_{56}$	0	0.25
β -Carotene, $\text{C}_{40}\text{H}_{56}$	0	0.25, 0.3
γ -Carotene, $\text{C}_{40}\text{H}_{56}$	1	0.95, 0.95
Lycopene, $\text{C}_{40}\text{H}_{56}$	2	1.5, 1.6, 1.6
Prolycopene, $\text{C}_{40}\text{H}_{56}$	2	1.65
Lutein, $\text{C}_{40}\text{H}_{56}\text{O}_2$	0	0.3

* It is interesting to note that the sum of one-half the average value (0.13) for carotenoids cyclised on both ends and one-half the average value (0.8) for fully aliphatic carotenoids is approximately equal to the value of the intermediate stage evidenced by γ -carotene (0.95).

SUMMARY

"Monkey flowers" (*Mimulus longiflorus* Grant, Scrophulariaceae) contain a mixture of carotenoids from which 45.5 mg. of crystallized γ -carotene, $\text{C}_{40}\text{H}_{56}$, per kg. of dry material has been isolated. Differences in the melting points of γ -carotene from various sources have been discussed.

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Quantitative Colorimetric Determination of Iron in Biological Material

p-tert-Butyl-o-nitrosophenol (Butylnitroso) a New Organic Reagent

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Butylnitroso is a new organic compound. It possesses properties which makes it valuable in chemical analysis. It is soluble in organic solvents with a pale green color. Many metals form highly colored, comparatively stable inner complex compounds with butylnitroso (1, 2). For instance, certain metallic ions (Fe, Co, Cu, Ni, Hg and others) can be selectively extracted from biological material by using a petrol ether or ligroin solution of butylnitroso under different working conditions. Colorimetric determinations of trace metals are especially valuable for routine analysis where simple and quick methods are required (5).

The quantitative determination of trace metals in bacteriological media becomes more and more urgent and important. For instance, the observation by Pappenheimer and Johnson (3) that abundant formation of diphtheria toxin occurs only in a relatively narrow zone of concentration of iron requires a quick iron determination on the medium in order to have constantly controllable working conditions. Other microorganisms like tetanus bacteria behave similarly. As it can be seen later, iron can easily be extracted and colorimetrically determined in such media with a ligroin solution of butylnitroso. For successful colorimetric determinations of merest traces of metallic ions in biological material, only absolutely pure and controllable organic reagents can be used. The preparation of pure butylnitroso is most simple and inexpensive. Its calcium salt is quite stable if kept under the described conditions.

The determination of traces of iron was first worked out with a standard iron solution using a photoelectric colorimeter (Pfaltz and Bauer,

New York, N. Y.). The curve is nearly a straight line. This means that the proposed method is entirely suitable for the colorimetric determination of iron, even with polychromatic light.

Procedure. Purified ligroin* is placed in both cuvettes and the colorimeter is set in the usual manner. The purity of the butylnitroso solution is then tested and the reagent repurified if necessary.

To the slightly acid iron solution, pH from 3.0 to 6.5, in a separatory funnel is added 3 to 10 mg. of *d*-isoascorbic acid if total iron is to be determined. The ferric iron present is thus quantitatively reduced to

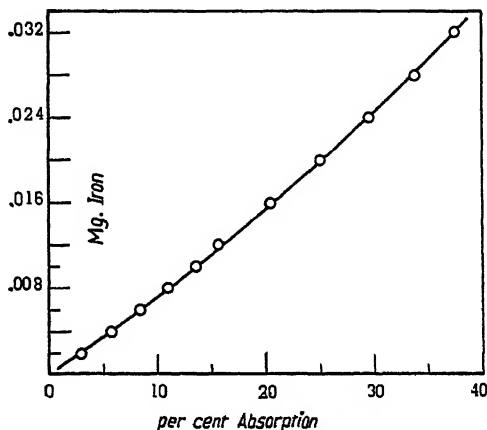


FIG. 1

ferrous iron. If only ferrous iron is to be determined, the addition of *d*-isoascorbic acid is not necessary. The solution is shaken well and 1 to 3 drops of pyridine are added (from 2 to 12 drops were added to different samples with no variation in final readings). The iron is then extracted from the solution by using a butylnitroso solution in purified ligroin. During the extraction the materials must be kept out of daylight as there is a possibility that the reagent may decompose.

Add an excess of butylnitroso in ligroin solution and shake vigorously for 20 to 30 seconds. The green, ligroin soluble iron complex forms immediately. The solution is allowed to stand a short time and the ligroin layer is separated and placed in a graduate. The procedure is repeated and then the water solution is washed with purified ligroin.

* Commercial ligroin (B.p. 90–120°) is shaken with concd. H_2SO_4 , washed with water until free of acid and distilled (B.p. 90–107°).

Add the ligroin to the green iron extraction in the graduate and bring the volume up to 12 ml. by adding ligroin.

The extraction mixture is then transferred to a separatory funnel and the excess butylnitroso is removed from the ligroin. This is done by adding 3 to 4 ml. cupric nitrate solution and shaking vigorously for 30 to 60 seconds. Separate the copper solution and repeat the extraction until there is no change in the color of the copper solution. Twice is usually sufficient. The green ligroin solution is then washed with 10 to 12 ml. of cold distilled water, twice with 10 to 12 ml. of cold water plus 3 to 5 drops of pyridine and once again with 10 to 12 ml. of cold water. After separation of the water the green ligroin solution is filtered directly into a cuvette for the colorimeter reading.

After the removal of excess butylnitroso the green iron complex is very stable in the absence of daylight. No change took place in from 48 to 72 hours in various tests. However, there was a slight change in iron salts that had been exposed to direct sunlight for eight hours.

The curve shows data obtained by using the above procedure. The normal sensitivity of the photoelectric colorimeter (Pfaltz & Bauer), 10 ml., 10 mm. cuvettes, a 15 C.P., 6-8 volt bulb and a 6 volt storage battery were used. No filters were used. In all cases the total volume of ligroin solution was 12 ml. The readings are those of the per cent absorption scale of the colorimeter plotted against the mg. of iron used. The curve is almost a straight line over the range shown.

Many different readings were made in which the conditions were varied. Different amounts of butylnitroso solution were added to the samples, the amount of pyridine added was changed, both in the original step and after the removal of copper, the pH of the iron solutions was changed, different dilutions of iron were used, and butylnitroso that had been made at different times was used. In all cases, if an excess of pure butylnitroso was added to an iron solution of pH from 3.0 to 6.5 the readings checked.

Under the conditions stated, two γ of iron as the green complex in twelve ml. of ligroin solution gave a reading of 3 per cent. The use of suitable color filters may increase the sensitivity.

PRACTICAL APPLICATION OF THE METHOD

Determination of Iron in Liquid, Biological Medium

Materials: Tetanus Medium. The pH was adjusted to 3.32 by adding hydrochloric acid as reduction by *d*-isoascorbic acid is complete from pH of 3.0 to 6.5.

d-Isoascorbic acid—U.S.P. (Pfizer).

Pyridine—C.P. (Merck).

Cupric Nitrate—10% aqueous solution.

Purified Ligroin.

Butylnitroso in purified ligroin solution.

Procedure. In the preliminary work on the medium it was found that after adding *d*-isoascorbic acid and pyridine the ferrous ions reacted quickly with butylnitroso to give the green iron complex. Various determinations were made using this reagent and the following procedure was found to be best.

Add 0.2 g. of *d*-isoascorbic acid to 200 ml. of medium and place in the icebox overnight. This is done to insure sufficient time for the complete reduction of ferric iron to the ferrous form. Then add 1.5-2 ml. of pyridine to the cold medium and shake well. Add 15 ml. of purified ligroin, shake well and separate. Filter the ligroin into a cuvette and test in the colorimeter. This will show whether there is any material in the medium which is soluble in ligroin. In the medium used here there was no ligroin soluble matter, therefore this step was only applied to the first sample.

The iron is then extracted from the medium by using a butylnitroso solution in purified ligroin. During the extraction and separation period the materials must be kept cold and out of daylight as there is a possibility that the reagent may decompose. Complete extraction of the iron is obtained by shaking the medium with 10 ml. of butylnitroso solution for 2 to 5 minutes and then allowing the separation to take place in the cold (30 minutes). The ligroin solution that separates after the first extraction is light green due to the formation of the iron complex. This is placed in a glass stoppered graduate and kept cold. The extraction is repeated again with 10 ml. butylnitroso in ligroin. Then the medium is washed twice with 5 ml. ligroin each time and after separation, this is added to the green iron extraction and the whole extraction mixture is then brought to a volume of 32 ml. by adding ligroin.

The extraction mixture is then transferred to a separatory funnel and the excess butylnitroso is removed from the ligroin by using the cupric nitrate wash as is described under the general procedure. The light green ligroin solution that is obtained is filtered directly into a cuvette for the colorimeter reading. The results obtained from work on three samples were as follows:

	Sample	<i>d</i> -Isoascorbic acid added	Pyridine added	γ of iron found	γ per liter
(1)	400 ml.	0 10 g.	2 0 ml.	16.66	41 6
(2)	200 ml.	0 50 g.	3.0 ml.	8.26	41.3
(3)	200 ml.	0 20 g.	1.5 ml.	8 26	41 3

In sample (1) the isoascorbic acid and pyridine were added only 5 minutes before the extraction took place. In addition, a butylnitroso solution that had been diluted 1:1 with ligroin was used. The complete extraction gave only 11.66 γ of iron. After 2 hours another complete extraction was made and 5 γ of iron were recovered. This gave a total of 16.66 γ for the 400 ml. sample of medium.

Qualitative determinations were made on the medium of sample (2), after the extraction of iron by butylnitroso. This was done by adding an aqueous solution containing 0.5 g. of neocupferron and allowing the precipitate to settle. The mixture was then extracted with chloroform to remove all the neocupferron (4). The extraction mixture was evaporated and ignited in a silica dish. Qualitative tests for iron were made on the residue. No iron was found.

SYNTHESIS AND PURIFICATION OF BUTYLNITROSO

Add 0.4 g. *p*-tert-butylphenol (E.K. Practical) to 10–15 ml. of ligroin and heat until the butylphenol has dissolved. Add this solution to 600 ml. of boiling distilled water and continue boiling until all the ligroin has evaporated. The butylphenol will then be in aqueous solution. Cool to room temperature and if there is any precipitate, filter the solution. Then add 4 g. hydroxylamine hydrochloride and 0.5 g. copper sulfate. If there is any precipitate at this point, filter the solution again and then add 2.0 ml. superoxol.

A few minutes after the addition of superoxol, the solution turns red due to the formation of the copper complex of butylnitroso. Let the reaction mixture A stand at room temperature for 2 to 2½ hours and then purify. With longer standing, more secondary products are obtained.

Purification Procedure. 10 to 15 ml. of hydrochloric acid (dilute 1:5), and 20–25 ml. of chloroform are added to A in a separatory funnel. Shake well and separate, then add more chloroform and continue the extraction until there is very little color in this solvent. The extraction mixture which contains the butylnitroso and some impurities, is then combined and washed with cold distilled water to remove the acid (test with congo red paper). The mixture is then

shaken with an aqueous solution of calcium hydroxide. The water layer becomes red-orange due to the formation of the water soluble calcium salt of butylnitroso. Separate the red water layer and repeat the extraction of the chloroform solution with calcium hydroxide solution until there is no more red color obtained in the water layer. Combine the red, water solutions of the calcium salt of butylnitroso and extract with chloroform until the chloroform is perfectly clear. Separate and filter the red water layer. At this point, all the chloroform should be removed as the presence of any chloroform in the final ligroin solution of butylnitroso will give a red color in the organic layer after the cupric nitrate wash and this will affect the colorimeter readings. The filtering of the water layer after separation usually removes the chloroform, if not, filter again.

Add 30 to 40 ml. of purified ligroin and enough hydrochloric acid to the water solution of calcium salt to make the solution acid, shake well and separate. At this point the ligroin is greenish due to the presence of butylnitroso. Repeat the extraction until there is no more green color obtained in the ligroin solution. The total volume of ligroin solution should be about 80 ml. after the complete extraction. Combine and wash the ligroin extraction mixture with cold distilled water until all the acid is removed. Separate and filter the ligroin solution on a filter wet with ligroin in order that most of the water is removed. This solution of butylnitroso is ready for use and will remain stable for seven to twelve days if kept cold and out of daylight. However, before using for quantitative work, its purity should be tested.

If the butylnitroso is not to be used for some time, add solid calcium hydroxide to the ligroin solution and let the red calcium salt of butylnitroso stand under the ligroin. In this form and under purified ligroin it will remain stable for a considerable length of time.

Test for Purity. To 12 ml. of butylnitroso solution in a separatory funnel, add 4 to 5 ml. of ten per cent cupric nitrate solution and shake vigorously for 30 to 60 seconds. The red, water soluble copper complex is formed with the reagent. Separate the copper solution and repeat the extraction until there is no change in the color of the copper solution. Twice is usually sufficient. The ligroin is then washed with 10 to 15 ml. of cold distilled water, twice with 10 to 15 ml. of cold water plus 3 to 5 drops of pyridine and once again with 10 to 15 ml. of cold water. After separation of the water the ligroin is filtered directly into a cuvette for the colorimeter reading. The per cent absorption reading should be zero. If not, repurify the butylnitroso by shaking with an aqueous calcium hydroxide solution to form a water solution of the calcium salt of butylnitroso. Then continue as in the usual purification procedure.

This test should be made just before any quantitative work is to be done.

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The Utilization of Fats by Herbivora

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INTRODUCTION

In earlier studies of fat utilization (McCay and Paul, 2) with guinea pigs, it was found that this species was more sensitive to the melting point of fats than any other known vertebrate. These investigations have now been extended with guinea pigs and preliminary ones have been made with rabbits and sheep.

Answers to the following questions have been sought:

1. How does the guinea pig compare with the rat in its utilization of oleic and elaidic acids? Can guinea pigs utilize ricinoleic acid? Is the degree of saturation or the melting point the more important in influencing utilization?

2. Are rabbits and sheep like guinea pigs in failing to utilize high-melting fats?

3. Can other herbivorous species digest and absorb castor oil like guinea pigs? Can guinea pigs utilize ricinoleic acid?

4. Can the milk fat from a cow, fed cod liver oil, destroy the vitamin E in the gastrointestinal tract of the guinea pig?

The experiments described here were preceded by a considerable number of preliminary trials with rabbits, sheep and guinea pigs that need not be described in detail. In the course of these, it was found that herbivorous animals can tolerate relatively high levels of fat in their diets. As much as 30% of the diet by weight may be made of fat and such diets can be fed to rabbits for 8 to 10 days without trouble. Sheep tend to refuse to eat after a few days feeding upon such high levels, but will consume diets containing ten per cent of fat for long periods. No evidence has ever been obtained that the body fat of sheep can be softened by long feeding upon low melting fats.

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Experimental

A mixture of two parts of calf meal (Maynard, 1) and one part alfalfa leaf meal was extracted for four days with isopropyl ether to remove lipids. This served as a basal diet for guinea pigs. Additional supplements of vitamins A and D as well as tomato juice were fed separately.

Elaidic acid was prepared by the method of Sinclair (3). The ricinoleic and oleic acids used were commercial products.

Sixteen young guinea pigs over 200 g. in weight were used for the first balance studies. To determine metabolic fat the feces were collected individually for seven days after the guinea pigs had been maintained upon the fat-low diet for a preliminary period of one week. The fat-low diet contained only 0.3% of ether soluble materials. The feces during this period contained a mean of 1.7% (Range 1.3–2.0%) of ether soluble materials.

Six of these animals were then fed the basal ration mixed with 6% of its weight of oleic acid and five were used for a similar trial with a diet containing 6% ricinoleic acid. After preliminary feeding of the diets for a week the feces were collected and the utilization calculated. 92–97% of the oleic acid was utilized and 91–93% of the ricinoleic.

Great difficulty was experienced in getting the guinea pigs to eat the diet containing ricinoleic acid and they died shortly after the second week of feeding. Some had developed paralyses similar to those found from feeding cod liver oil. In the light of recent experience this may have been due to vitamin E deficiency.

Before making the trials to compare elaidic and oleic acids the diets were improved by including 0.3% alfalfa leaf meal five mg. of ascorbic acid, and 0.01% irradiated yeast in the diet. Excess of A-D concentrate was fed separately during recovery periods.

After a preliminary period upon this diet six guinea pigs were fed the diet mixed with 6% elaidic acid. After five days upon this diet, the balance was started and continued for seven days. The same animals were then changed first to the normal diet, then for a preliminary period of five days upon the mixture with 6% oleic acid and finally a balance lasting seven days was run again using oleic acid.

The per cent of elaidic acid utilized varied among individuals from 44 to 69, with a mean of 56. The corresponding values for oleic acid were 93 to 96 with a mean of 95. The metabolic fat was again determined and found to be 1.2% of the dry weight of the feces. At this time the mean weight of the guinea pigs was about 500 g. and the metabolic fat

was lower than the 1.7% found three months earlier when the animals weighted only half as much.

These data are summarized in Table I.

THE UTILIZATION OF OLEIC AND ELAIDIC ACID BY RATS

In order to determine the relative utilization of oleic and elaidic acids by rats, three diets were fed, constituted as shown in Table II.

To supply water soluble vitamins a small allowance of rice polish concentrate was also given. Fat soluble ones as well as essential fatty

TABLE I
Utilization of Fatty Acids by Guinea Pigs

Diet	Per cent utilized
Oleic acid (6%).....	94.6 \pm 0.55
Elaidic acid (6%).....	55.6 \pm 2.54
Oleic acid (6%).....	95.4 \pm 0.27

TABLE II

	No. I	No. II	No. III
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	20.0	20.0	20.0
Salt mixture (O. and M.).....	4.5	4.5	4.5
Sucrose.....	65.0	65.0	75.0
"Vitab" concentrate.....	0.5	0.5	0.5
Oleic acid.....	10.0		
Elaidic acid.....		10.0	

acids were fed separately in an amount of 0.05 cc. daily per rat, of a fish liver oil concentrate in linseed oil.

Five rats weighing 57 to 74 g. were used in each balance. Upon the diets containing fatty acids, the fecal collections covered six days after a preliminary period of four days upon the diet. A collection period of thirteen days after a preliminary one of seven days was used to determine metabolic fat, feeding diet III.

The results are summarized in Table III.

The rat in contrast to the guinea pigs makes equal use of the two isomers. The chief observation of interest in this study was the greater

weight of dry matter in the feces of rats fed elaidic acid. Very large pellets were excreted by these animals.

THE UTILIZATION OF FATS OF DIFFERENT MELTING POINTS BY RABBITS

To compare rabbits and guinea pigs a balance study was made, starting with a feed mixture of alfalfa 80, crushed oats 10, and crushed wheat 10. This was extracted with isopropyl ether. Vitamins A and D were supplied in one drop of high potency concentrate fed every other day. The fats, added to this at 6% levels, were cottonseed oil, hydrogenated oil (Crisco) and castor oil.

TABLE III
Utilization of Elaidic and Oleic Acids by Rats

Diet	Mean daily gain in wt.	Food eaten daily	Mean daily wt. dry feces	Utilization of fatty acids
	grams	grams	grams	per cent
III, fat low.....	1.05	6.2	0.12	
I, oleic acid.....	0.85	5.4	0.17	95.4 \pm 0.81
II, elaidic acid.....	0.93	5.9	0.38	95.6 \pm 0.61

TABLE IV
The Utilization of Soft and Hard Fats by Rabbits

Diet	Per cent utilization (uncorrected for metabolic fat)
6% cottonseed oil.....	91.2
6% hydrogenated (Crisco).....	91.0
6% castor oil.....	92.1

Two rabbits weighing about 3 kg. were used for the balance studies. After a preliminary feeding of the fat low ration for eight days, the collection period lasted for an equal time. The fat per cent in the feces was 2.2% for both rabbits.

In diets containing fats the period of feeding was extended to eighteen days with fecal collections the last ten days, since it was recognized that the habit of the rabbit in eating night-feces makes a long preliminary period essential. The results are summarized in Table IV.

These data indicate that the rabbit compares favorably with the guinea pig in its efficient utilization of castor oil, but resembles the rat in its ability to make good use of high melting fats.

FAT UTILIZATION BY SHEEP

Various studies of fat utilization have been performed with sheep, in our laboratory. Since they have given the same results in regard to the utilization of hard fats, only one typical case is included in this report.

A 30 kg. lamb was placed in a metabolism cage and fed a normal diet of alfalfa meal, 79, wheat bran 10, rolled oats 10, limestone 0.5, and salt 0.5.

Balances were run during a series of periods, feeding the basal diet, the basal extracted with isopropyl ether, the extracted diet supplemented with 6% cottonseed oil, with 6% Crisco and with 6% castor oil. Vitamin concentrate of A and D was used whenever the extracted diet was fed.

Four-day preliminary and seven-day balance periods were used in each trial. The feces were normal in all cases except that they were

TABLE V
The Utilization of Fats by Sheep

Diet	Per cent utilized
Normal ration.....	54
6% Cottonseed oil.....	94
6% hydrogenated (Crisco).....	94
6% castor oil.....	99

slightly soft during the preliminary feeding of the low fat diet. The results are summarized in Table V.

This study as well as others in which such hard fats as mutton tallow have been fed to sheep indicates that sheep and rabbits resemble omnivorous species rather than guinea pigs in their utilization of fats.

The poor utilization of the ether soluble material in the normal ration was probably due to its high content of chlorophyll. Horwitt, Cowgill and Mendel (4) have pointed out that the chlorophyll in the ether extract of leafy materials is not absorbed. In the green leaf they found only 2.2% was made up of true fats while 5.5% of the dry leaf was soluble in ether.

THE EFFECT UPON GUINEA PIGS OF FEEDING MILK-FAT SECRETED BY COWS FED COD LIVER OIL

Cows fed cod liver oil secrete a lower per cent of fat in their milk but this fat has a higher iodine number (McCay, et al., 6). Hilditch and

Thompson (5) found an increase of unsaturated fatty acids from about 1% in normal butter to 5-8% in that from cows fed cod liver oil.

Cod liver oil also produces degenerative changes in the muscles of herbivora such as sheep, rabbits and guinea pigs due in part, at least, to the destruction of vitamin E in the gastro intestinal tract.

While the study of the utilization of fats by guinea pigs was in progress, supplies of fat secreted by cows fed cod liver oil became available. These afforded an opportunity to determine if the components of cod liver oil responsible for the production of muscular dystrophy, passed over into the fat secreted by the cow.

Cream was obtained from three cows after they had been fed six ounces of cod liver oil daily for two weeks. At the same time cream was taken from three cows not fed cod liver oil. This cream was churned into butter before feeding to guinea pigs.

Twenty guinea pigs were divided into two groups. They were fed a basal diet of casein 15, sugar 15, starch 31, cellulose 15, agar 5, yeast 7 and salt mixture 4. Tomato juice was fed in addition at the rate of 2 ml. per 100 g. of live weight per day. Eight parts of the butter fats were added for the two groups in order to compare normal milk fat and that from cows fed cod liver oil.

The young guinea pigs were started when they weighed 250 g. and their growth rates were similar for 100 days. Three members of each group were killed after 50 and 70 days to determine if there was any evidence of the degeneration of the muscles.

Evidence of muscular dystrophy commonly found in guinea pigs fed cod liver oil appeared in only one case and this was in the group fed normal butter fat.

These results indicate that the butter fat from cows fed cod liver oil does not promote the degeneration of muscles in guinea pigs. Furthermore, the cream from a cow fed cod liver oil was placed in cold storage and fed to the cow after a recovery period to allow the milk fat secretion to return to normal. This feeding of cream caused no change in milk fat secretion. This indicates that the factor of cod liver oil that causes the decreased secretion of milk fat as well as that which destroys vitamin E and produces muscular dystrophy in guinea pigs does not pass through into the milk fat.

SUMMARY

Diets containing as much as 30% of fat can be fed to guinea pigs. The melting point of a fat is important in determining its utilization

by guinea pigs but not in the case of rabbits and sheep. Guinea pigs only utilize about half as much elaidic acid as oleic acid when these isomers are fed as 6% of the diet. Rats utilize the isomers equally well. In contrast to guinea pigs, rabbits and sheep digest and absorb hard fats as well as soft ones.

The milk fat, secreted by cows fed cod liver oil, was well tolerated by guinea pigs. No lesions of the muscles resulted, so it is unlikely that the fraction of cod liver oil that leads to the destruction of vitamin E, is secreted by a cow fed cod liver oil.

Rabbits resemble guinea pigs in being able to absorb castor oil. Melting point rather than degree of saturation seems to be the determining factor in the utilization of fats by guinea pigs.

A cow fed milk-fat, secreted during a period of cod liver oil feeding, remained normal, indicating that the factor responsible for depressing the secretion of fat does not pass into the milk, from the cod liver oil.

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The Biochemistry of Autotrophic Bacteria

The Metabolism of *Thiobacillus thiooxidans* in the Absence of Oxidizable Sulfur

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Autotrophic bacteria have the unique property of living entirely upon inorganic elements or simple inorganic compounds. These they oxidize for energy and synthesize their cell material from carbon dioxide. The strictly autotrophic bacteria, of which *Thiobacillus thiooxidans* is a well known example, are able to derive energy only from the oxidation of a specific nutrient (in this organism sulfur is oxidized to sulfuric acid) and to derive their carbon only from carbon dioxide, by a process carried on in the dark known as "chemosynthesis." *Thiobacillus thiooxidans* has still more unusual properties in that it grows in solutions containing as much as 5% sulphuric acid.

The importance of studies on this apparently unique form of life from the viewpoint of comparative biochemistry has been previously discussed (1). It was recently shown by Vogler (2) for this organism and by Bomeke (3) for other autotrophic bacteria (*Nitrosomonas*, *Nitrobacter*) that contrary to earlier conceptions, the organisms possess an organic metabolism independent of the presence of the specific nutrient. *Th. thiooxidans*, for example, while strictly dependent upon sulfur (or thio-sulfate) and carbon dioxide for growth, is able to respire and to produce carbon dioxide from its cell substance in the absence of even traces of oxidizable sulfur, once the cell has grown. The nature of the metabolism of the autotrophic cell in the absence of specific nutrient is the subject of this paper.

The problem of the metabolism of the autotrophic cell itself is some-

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what difficult to approach inasmuch as these organisms are not able to transform external supplies of organic matter (4); one cannot, for example, add a sugar and determine the products of fermentation. A different method of study was necessary. This consisted of a search for storage materials, proof that storage materials found were active in the metabolism, and some indications of the pathway through which the storage materials were broken down. The first problem was to determine whether the endogenous respiration observed by Vogler (2) resulted from the breakdown of a more or less specific material (comparable to starch or glycogen) or whether various parts of the cell substance were broken down. It was known that the organism was high in fat (5), but microscopic observation indicated that the fat did not disappear on long incubation in the absence of sulfur. It was also known that the endogenous respiration was rather slow, and that the energy derived from it was not sufficient to permit carbon dioxide fixation (6) and hence growth. It seemed to be more of a survival mechanism which could enable the organism to live in soil during periods in which sulfur was not available.

STORAGE MATERIAL

In a search for materials which might serve in this organism in a manner similar to starch and glycogen in heterotrophic organisms, O'Kane (7) was able to isolate a polysaccharide material by the glycogen method of Good *et al.* (8), but soon found that the material was not glycogen. There was no evidence that this material played an active role in the metabolism. Proof that it actually has a storage function, and that its breakdown is responsible for a large part of the carbon dioxide liberated during respiration in the absence of sulfur was obtained in the following manner:

The autotrophic cells were grown under pure culture conditions as previously described (5), with a plentiful supply of sulfur. They were harvested at six days to provide "young" cells (6) by centrifuging in a Sharples steam-driven super-centrifuge, washed once in distilled water, and freed of the last traces of sulfur by filtration through paper pulp. Nitrogen was determined by a method similar to that of Johnson (9) and the suspension made to 300 micrograms bacterial nitrogen per milliliter. A trace of inorganic phosphate was added, and sulfuric acid to give a final pH of 2.0 since the *Thiobacillus* is not influenced by this low pH while any contaminants which may have entered during the harvesting process would probably be inhibited. The usual contaminant found in these suspensions is a red *Torula*. It sometimes appears in the growing cultures, but usually enters

during the harvesting process. In these experiments particular precautions were taken to prevent the entrance of this contaminant. The suspension was examined frequently during the experiment for contaminants and appeared free of them even after 48 hours. Thus contaminants are not responsible for the carbon dioxide production or other changes noted. In the experiment reported, 450 ml. of the suspension were placed in an aeration apparatus and aerated with CO_2 -free air. The CO_2 produced was absorbed in two absorbers containing standard barium hydroxide and its quantity estimated by titrating the residual barium hydroxide with standard oxalic acid. Aliquots were withdrawn from the suspension at intervals for measurement of cell activity on sulfur, amount of polysaccharide, inorganic phosphorus in the medium, total (i.e., organic) phosphorus in cells and medium, total number of cells and number of viable cells. For the determination of polysaccharide the method of Good *et al.* (8) was used except that total carbon was determined on the purified polysaccharide rather than reducing sugar. In the later stages of the experiment some polysaccharide appeared in the suspending medium. This was estimated as above after first concentrating the medium to a small volume with vacuum distillation. Total carbon was determined by wet combustion with chromic acid in the following manner: samples of the purified polysaccharide solution were placed in Erlenmeyer flasks with chromic acid solution (composition that of Schollenberger, 10). Samples and blanks were heated on a hot plate for 5 minutes, cooled, diluted with distilled water and supplied a CO_2 atmosphere by the addition of a small quantity of NaHCO_3 . A measured excess of ferrous ammonium sulfate was added to each sample and the excess determined by titration with standard potassium permanganate. Inorganic phosphorus was determined by a modification of the Fiske and Subbarrow method (11), employing the Evelyn photoelectric colorimeter as follows: the samples (containing from 10 to 100 micrograms PO_4 phosphorus) were placed in 19×155 mm. tubes graduated at 25 ml. These had been selected for uniform light transmission. To each sample was added 1 ml. 10 *N* H_2SO_4 , 2 ml. of 2.5% ammonium molybdate and water to about 20 ml. One ml. of the Fiske and Subbarrow reducing agent was added and the volume made to 25 ml. After 10 minutes the color was measured with the Evelyn photoelectric colorimeter, using a 6600 Å. filter. A standard was included with each set. The color follows Beer's law over the range 10–100 micrograms, allowing a precision of ± 0.4 micrograms, duplicates usually agreeing to within 0.2 micrograms. Organic phosphorus compounds do not react under these conditions, with the exception of creatine phosphate, which is absent from this organism. Total phosphorus was determined as inorganic after digestion with sulfuric acid and H_2O_2 . Extraction of phosphorus compounds from the cells for a fractionation was accomplished by dissolving the cells in 2 *N* NaOH (while this destroys the triosephosphates, it is the only method by which any quantity of organic phosphorus can be extracted) and extracting the alkali-treated cells overnight with 10% trichloroacetic acid. Barium fractionations were made as described elsewhere (12) and hydrolysis curves were obtained at 100°C. in 1 *N* HCl . Oxygen uptake on sulfur was determined by adding an aliquot of the cell suspension containing 45 micrograms bacterial nitrogen to a Warburg flask with an excess of sulfur in the presence of $M/60 \text{ KH}_2\text{PO}_4$ (pH 4.5) using KOH in the center cup. This was equilibrated 4.5 hours to allow sulfur-

bacterial contact to be established (1) and oxygen uptake was determined. *Total cell counts* were determined by the method of Breed (13) and *viable cell counts* were determined by dilution counts and the "most probable number" (14) calculated. The medium used was that described by Waksman and Starkey (15) employing inorganic salts and sulfur. Growth was determined by turbidity.

For purpose of brevity, only one experiment will be described. Essentially the same results were obtained in three other experiments.

The data obtained are recorded in Table I. It may first be noted (Columns 1, 2, 3, 4) that the drop in polysaccharide content accounts for all the carbon dioxide produced. Indeed, the drop in polysaccharide content in the earlier stages of the metabolism is greater than needed to account for the carbon dioxide production, indicating some of the polysaccharide has been converted to metabolic intermediates. Thus the polysaccharide is a storage material whose metabolism is responsible for the endogenous respiration. During the oxidation of sulfur this polysaccharide is presumably formed and stored in the cells for use when sulfur is absent.

NATURE OF THE POLYSACCHARIDE

It was therefore of some interest to determine the nature of the polysaccharide material. Accordingly samples were prepared from several batches of cells. When such material was further purified by repeated precipitation and dialysis against distilled water, the purified materials had the following composition (ash-free basis for carbon, hydrogen):

C	43.25% (dry combustion),
H	7.86% (dry combustion),
O	49.89% (by difference),
P	0.68-1.65%,
N	0.82%.

Further purification did not result in a lowered phosphorus or nitrogen content. The range shown for phosphorus content represents a variability in this with different samples. The other percentages here given are for a sample containing 0.68% P.

The polysaccharide was soluble in water and quantitatively precipitated by addition of 1 part of 95% ethanol, but only if electrolytes were present. On hydrolysis at 100°C. in 1 N HCl it gave maximum reducing value with sugar reagent (16) after 2.0 hours, thus being slightly more resistant than a glycogen, which was completely hydrolyzed in 1.5

hours under these conditions. The maximum reducing activity calculated as hexose only accounts for 50% of the compound.

Investigations using the carbazole reaction of Gurin and Hood (17) indicated the polysaccharide contained either an equimolar mixture of glucose and mannose, or galactose alone. The phenylosazone obtained with the hydrolyzate of the polysaccharide was that of glucose, which would of course be the same for mannose. A need for prolonging reac-

TABLE I
*Relationship between Loss in Polysaccharide and CO₂ Production
in Thiobacillus thiooxidans*

Time	Total CO ₂ produced mg /g dry wt of cells	Polysaccharide			Phosphorus		QO ₂ (N) on sulfur	Bacterial counts		
		mg /g dry wt of cells	Loss in fraction mg /g. dry wt of cells	% of CO ₂ accounted for by loss in polysac- charide	Inor- ganic	Organic in solu- tion		Direct (total no. of organ- isms) × 10 ¹⁰ per ml.	Living cells	
									Dilution count × 10 ¹⁰ per ml	
									1 week	2 weeks§
	1	2	3	4	5	6	7	8	9	10
<i>hours</i>					<i>γ/ml.</i>	<i>γ/ml</i>				
0	0	124.5	0	0	21.20	3.00	3,500	15.6	3.5	16.0
2					22.30	3.35				
4	2.77				23.05	4.15				
6					24.20	5.60				
8	5.95	119.3	5.20	126.0	26.35	4.65	2,780	16.5	2.5	6.9
10					28.75	4.75				
12	9.30				29.15	5.80	2,030			
24	13.68	115.0*	9.50	101.0	34.15	5.60	1,290	16.6	0.7	6.0
32	16.87				40.25	5.50	863			
48	45.07	95.3†	29.20	93.5	40.50	6.40	550	7.3‡	0.01	5.0

* 8% in the supernatant fluid.

† 18% in the supernatant fluid.

‡ Badly clumped.

§ No change in count at 3 weeks.

tion time with the sugar reagent to get complete reaction further indicated the presence of mannose. Hence it would seem the material contains glucose, mannose and some further reduced material.

PHOSPHORYLATION

The next question which arises is how the polysaccharide is metabolized. Does it, for example, follow a phosphorylating "glycolysis" as

do yeast and muscle, or is there a different method of degradation? Presumptive evidence that phosphorylation is involved in the decomposition of the polysaccharide is the release of inorganic phosphorus into the medium (Table I, Column 5). Since there is a drop in the number of viable cells (Column 10) this phosphate release might be due merely to autolysis of dead cells. That this is actually not the case is apparent for two reasons. First, the amount of organic phosphorus in the suspending medium (Column 6) shows essentially no change. If cell autolysis were occurring, one would expect this value to increase markedly. Second, the number of visible organisms determined by direct count is, within limits of experimental error, also unchanged (Column 8). It may therefore be concluded that the release of inorganic phosphorus shown in Column 5, for the earlier part of the experiment at least, is related to the endogenous metabolism. The actual phosphorus release represents a rather large proportion of the organic phosphorus originally available (7.9% at 8 hrs., 19.9% at 24 hrs., 29.7% at 48 hrs.).

It would be of interest to know the types of compounds involved in the decomposition of the polysaccharide and work is being continued in this direction. The preliminary experiments of O'Kane have shown that many of the compounds are similar to, if not identical with, those found in heterotrophic systems.

The continued release of inorganic phosphorus during the endogenous respiration does not on the surface present a picture analogous to that which occurs in yeast or muscle juice, and certainly it is not analogous to that which can sometimes be demonstrated in living heterotrophic cells, where the changes are generally too small to be readily detected. If the polysaccharide were degraded by means of a series of phosphorylated esters, and if the initial steps in its degradation involved a phosphorylation as with starch or glycogen, one would expect no overall phosphate change. However, if the phosphate esters were in predominance and were decomposed more rapidly than they were re-formed from the polysaccharide, then the phosphate changes recorded would be more logical.

Analyses of the organic phosphorus of cells at the beginning and end of the experiment are given in Table II. The methods were those described by O'Kane and Umbreit (12). Aliquotes of the cells at the beginning and after the 48 hr. aeration were centrifuged down, treated with alkali, extracted with 10% trichloroacetic acid and the extracts fractionated. The inorganic phosphorus which had been liberated into the medium was, of course, removed and does not appear in these frac-

tions. The ratio of inorganic to organic phosphate in the cells is the same at the end of the experiment as at the start; the difference is in the amount of organic phosphorus available, which has decreased markedly. Other experiments checking the amounts of acid extractable phosphorus show that the inorganic phosphorus released into the medium during endogenous respiration is all accounted for by the decrease in acid extractable phosphorus of the cell. The changes in the barium insoluble

TABLE II
Phosphorus Distributions in Thiobacillus thiooxidans

	Cells at zero hour		Cells at 48 hours	
	γ	Per cent	γ	Per cent
Total phosphorus:				
Organic.....	4,520	86.0	1,814	86.0
Inorganic.....	740	14.0	282	14.0
Barium insoluble fraction:				
Organic.....	1,318	29.0*	760	41.8*
Inorganic.....	632		216	
Barium soluble fraction:				
Organic.....	2,290	50.6*	970	53.5*
Inorganic.....	0	0	0	0
Fractionation of barium insoluble phosphorus:				
Easily hydrolyzed $\Delta 7$		52.2†		27.6†
Resistant 100%— $\Delta 180$		0†		70.4†
Other.....		47.8†		0†
Recoveries:				
Organic.....	3,608	80.0	1,730	96.0
Inorganic.....	632	85.0	216	76.0

* Per cent of total organic.

† Per cent of total organic in fraction.

fraction are quite striking. Initially there is a considerable amount of readily hydrolyzable material (7 min., 1 N HCl, 100°C.), presumably adenosine tri-phosphate, with very little if any resistant phosphorus (not hydrolyzed in 180 min.), presumably phosphoglyceric acid. After 48 hours' aeration the easily hydrolyzable material has markedly decreased and most of the fraction is highly resistant to hydrolysis. These results are what one would expect if the ester phosphate were responsible

for the release of inorganic phosphorus observed. It remains to be determined whether the degradation is by a path similar to that in yeast and muscle juice. The barium soluble fraction is virtually all (95%) precipitable with alcohol in both cases, *i.e.*, resembles the hexosemonophosphates of the heterotrophic tissues.

One further point is of interest. The $\text{QO}_2(\text{N})$ on sulfur (Table I, Column 7) drops off quite rapidly, while numbers of cells (Column 10) drop more slowly. Apparently as the cells age under these conditions, the ability to grow is maintained even though the cells are not able to oxidize sulfur at as rapid a rate when placed in contact with it. There is a possible explanation available for this phenomenon. The organic acids, pyruvic, lactic, fumaric, all inhibit sulfur oxidation markedly (1, 6). If these or similar materials were accumulating in the cells due to the endogenous metabolism, they would give this effect of inhibited sulfur oxidation, but would not prevent growth when the cells were put in the new medium for dilution count.

SUMMARY

The organic metabolism of the autotrophic cells of *Thiobacillus thiooxidans* consists of a degradation of a polysaccharide storage material formed during the growth on sulfur. The properties of the polysaccharide are described. Phosphorylated intermediates appear to be involved in the breakdown of the storage material.

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The Digestibility of Some High Protein Feeds by Foxes

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The literature contains few reports of the digestibility of feeds by foxes. Inman and Smith (1941) studied the digestibility of beef and horsemeat by foxes. The average percentage digestibility of the protein of fresh beef was found to be 97.8, frozen beef 96.9, and frozen horsemeat 94.4. These differences may have been due to varying amounts of connective tissue. In a further study Inman (1941) compared the digestibility of frozen beef tripe, lip meat, beef hearts and cow udders. The digestibility of the protein of udders was lower than for the other three meats among which there was no significant difference. In the above studies the product under consideration was fed alone. Bernard, Smith, and Maynard (1942) published results of studies of the digestion of cereals by foxes with special reference to starch and crude fiber. In general the starch of cooked cereals was digested 90 per cent or better, whereas the starch of raw cereals was less digested. In these studies simple diets were fed consisting of frozen horsemeat, the cereal under consideration, tiki-tiki (a vitamin B concentrate) and cod liver oil.

The studies reported in the following pages were designed to gain further knowledge concerning the value of some high protein feeds as partial substitutes for raw meat in the diets of foxes. These desiccated feeds are much less expensive than raw meat, and they do not require expensive refrigeration. The maximum use of these feeds would thus materially reduce the cost of production.

PROCEDURE

A total of seven adult silver foxes were used in the digestion trials. Three foxes were used in each of seven trials which extended from December until the following March. The trials were conducted in

metabolism cages so designed as to permit a separation of feces and urine. Each trial consisted of a three-day preliminary period of adjustment and a five-day fecal collection period. The feces were gathered daily and preserved in acid-alcohol (1 part HCl to 99 parts of 95 per cent ethyl alcohol). The diets fed were thoroughly ground and mixed at the start of the trial. They were kept in a refrigerator and sampled at the beginning, middle and end of each fecal collection period. Each fox was fed 250 g. of the wet diet each day. Refused or wasted feed was collected, dried, and weighed back. Consumption, in general, was excellent. The diet containing blood meal and more particularly the one

TABLE I
Chemical Composition of the Diets Used

Description	Crude Protein	Composition on a Moisture-Free Basis			
		Ether Extract	Crude Fiber	Nitrogen-Free Extract	Ash
	%	%	%	%	%
Horsemeat	37.00	8.56	2.08	49.89	2.47
Meat scrap.....	31.88	12.61	1.72	45.84	7.95
Fish meal.....	32.04	8.64	2.01	51.32	5.98
Liver meal.....	34.51	11.26	4.99	46.41	2.83
Blood meal.....	37.71	6.17	3.21	51.67	0.24
Soybean meal.....	35.27	5.47	3.35	52.97	2.94
Linseed meal.....	30.39	5.20	3.27	57.88	3.26

containing soybean meal were most palatable and fecal form was excellent.

Dietary and fecal samples were dried at approximately 70°C., ground and analyzed for dry matter, crude protein ($N \times 6.25$), ether extract, crude fiber and ash by A.O.A.C. (1930) methods.

The plan of study was to compare the digestibility of the protein of a simple, adequate diet when the chief source of protein was varied. The basal diet had frozen horsemeat as the chief source of protein. The diets of comparison had approximately one-half of the protein of horsemeat replaced by meat scrap, fish meal, liver meal, blood meal, soybean meal, and linseed meal. Mixed diets were used, because some of the feeds under consideration were unsuitable for feeding alone. Also, it was desired to study the digestibility of protein under practical feeding

conditions where mixed diets are fed and where the associative effect of feeds, if present, would be expressed.

The basal diet had a percentage composition of frozen horsemeat 55, oat meal 30, tiki-tiki 0.5, reinforced cod liver oil 0.2 and water 14.3. The remaining diets were similar except that the horsemeat was decreased to 25 per cent, and 10 per cent of meat scrap, fish meal, liver meal, blood meal, 12 per cent of soybean meal or linseed meal were added respectively. Water was added to bring the percentage composition to 100. It was estimated that the foregoing feeds would replace about one-half of the protein of horsemeat in the basal diet. In the basal diet the protein of horsemeat made up about 75 per cent of the total protein and the oat meal about 25 per cent. The meat scrap had a protein guarantee of 55 per cent. The fish meal was vacuum dried whiting. The liver meal was prepared by the dry rendered vacuum process. The soybean meal was extracted by the solvent, and the linseed meal by the expeller process. The linseed meal contained 4 per cent of ether extract.

A summary of the chemical composition of the various diets is given in Table I.

RESULTS

This study was designed primarily to compare the digestibility* of various proteins. The coefficients of digestibility of the protein as well as ether extract and nitrogen-free extract are given in Table II.

The data were subjected to an analysis of variance and the least difference for significance determined. There was no significant difference between foxes. The digestibility of the protein combination of blood meal-horsemeat-oat meal and linseed meal-horsemeat-oat meal was highly significantly lower than that of horsemeat-oat meal which differences must be attributed to the lower digestibility of the protein of blood meal and linseed meal respectively. Also, the protein combination of meat scrap-horsemeat-oat meal and soybean meal-horsemeat-oat meal was significantly lower than that of horsemeat-oat meal. These differences are attributable to the lower digestibility of the protein of meat scrap and soybean meal respectively. The protein combinations of fish meal or liver meal-horsemeat-oatmeal were not significantly different from that of horsemeat-oat meal. This is not to be interpreted as indicating that the protein of fish meal or liver meal is as highly digested as that of frozen horsemeat but merely that under the conditions here outlined the protein digestibilities were not sufficiently different to

be significant. A comparison of diets containing larger proportions of fish meal and liver meal may show a significant difference from horsemeat.

The difference in digestibility of the ether extract in the various diets was significant only for the diet which contained linseed meal in which case a lower apparent digestibility was noted. Whether this is a true difference or an artefact secured by following the usual ether extraction cannot be answered now. It may be that the oil of linseed meal (a drying oil) when dried was incompletely extracted, giving false values. Likewise, there was no significant difference between the nitrogen-free extract values except for that furnished by the linseed meal diet and the

TABLE II

The Digestibility of Protein, Ether Extract, and Nitrogen-Free Extract

Diet	Digestibility of Crude Protein	Significance of Difference*	Ether Extract	Nitrogen-Free Extract
	%		%	%
Horsemeat	91.0	19:1	91.4	88.0
Meat scrap.	85.8		96.2	89.1
Fish meal.....	88.0		91.4	92.1
Liver meal.....	87.9		87.7	87.7
Blood meal.	77.7	99:1	86.8	84.4
Soybean meal.....	85.8	19:1	83.3	83.6
Linseed meal.	80.8	99:1	69.3	73.4

* As compared to the horsemeat basal diet.

Each figure is the average of three foxes.

true significance of this is doubtful. Since nitrogen-free extract is obtained by difference, any error in the determinations of the other dietary fractions would be reflected here, such as may have been the case in the determination of ether extract in the diet.

The values obtained for the apparent digestibility of crude fiber are not given, since they are of doubtful significance in these diets which contain such a small fraction of fiber.

DISCUSSION

The foregoing results show clearly that the protein of blood meal, linseed meal, meat scrap and soybean meal is of a lower digestibility than that of frozen horsemeat, the former two being lower than the latter

two. The design of this study did not show a difference of digestibility of protein between frozen horsemeat and fish meal or liver meal.

Since all of these desiccated feeds are less expensive than horsemeat, their use as a partial substitute for horsemeat would lower feeding costs. From the standpoint of digestibility at least, fish meal and liver meal would appear to be particularly worthy of further consideration as a raw meat substitute for foxes. While meat scrap and soybean oil meal furnish protein of a lower digestibility, they still are high enough to warrant further consideration. The good results obtained by Bassett (1941) in feeding foxes soybean meal would support this statement. The fact that soybean meal is the least expensive of all these feeds at present is an additional factor to be considered.

SUMMARY

By means of digestion trials with adult foxes it has been shown that the protein of blood meal, linseed oil meal, meat scrap and soybean oil meal is less digested than the protein of frozen horsemeat, the former two being less digested than the latter two. Under the conditions of this experiment the protein of liver meal and fish meal was not significantly different in digestibility than the protein of frozen horsemeat.

As a partial substitute for the raw meat in the diet of foxes, liver meal and fish meal and to a more limited extent meat scrap and soybean oil meal are worthy of further consideration.

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Weather and Biochemical Variability

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PART I

The material presented in this paper relates to the problem of organic variability as it finds expression in changing biochemical levels in the blood and the urine, in the water balance, in organ function, in resistance to intoxication or infection.

While the biochemist accepts certain biochemical variations (season, etc.) for practical purposes such changes are ignored or obscured by reference to theoretical 'means' or the citation of 'standard deviations' [see Vaihinger, "The Philosophy of AS IF" (1) as well as Lawrence Henderson's "The Study of Man" (2)] thereby evading the issue of what causes such pendulation—such 'standard deviation.'

For the past decade the effort has been made (3) to establish the significance of changing atmospheric environment as causal in such phenomena and in the first part of this study I shall merely depict the magnitude of the chemical changes that occur during the course of such adjustment to meteorological change in perfectly normal young adult male triplets living under normal conditions of activity and diet, in the unstable atmospheric environment such as that provided in the mid-continental region of America.

Observations were made at the same time every day for a period of six weeks. Blood was drawn from the arm.¹

¹ I would stress the importance of venous blood for this purpose, rather than arterial blood. Venous blood mirrors the condition of the skin and the skin in turn is the major organ which must mediate the environment (apart from the skin and the mucous membranes of the respiratory tract, sensory impulses from the eye and the ear may play a rôle in the mechanism of adjustment). Obviously the chemical changes will vary more than those observed in arterial blood, which represents an oxygenated and pooled sample. An ideal system would be the determination of arterial-venous difference, but for practical purposes this was not possible.

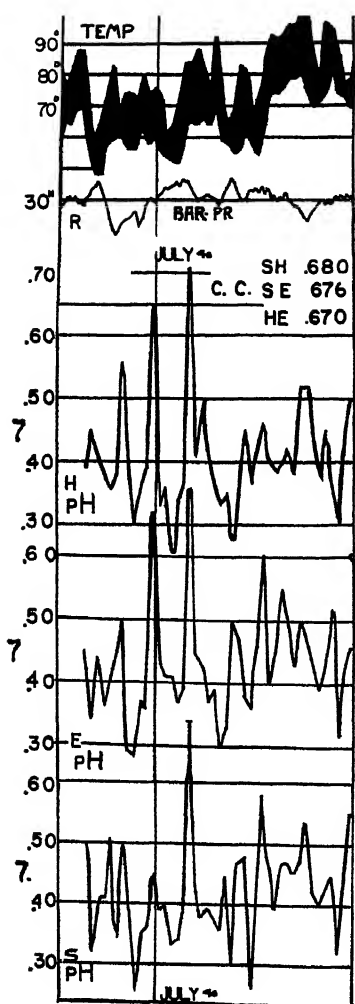


FIG. 1. Daily pH Determinations on the Venous Blood of the Arm of the Triplet Subjects H, E, and S.

Meteorogram is superimposed—maximal and minimal temperatures (black field) and barometric pressure.

The Blood pH

In Fig. 1 the daily pH determinations² of the venous blood from the arm for the three subjects (H, E, and S) beginning June 19 and continuing until August 1, 1940, have been graphed. Over the pH curves a meteorogram of the time has been extended. This indicates the daily maximal and minimal temperature, the barometric pressure, and rain-fall. Correlation coefficients indicating the degree of reactive concordance between Subjects S and H, S and E, and H and E are .680, .676, and .670 (significance being about .3).

² The pH determinations were made on blood plasma by the glass electrode method. The buffer solution was compared at weekly intervals with a standard pH solution. To assure constancy of our standard pH solutions they were checked every six weeks against numerous precision hydrogen electrode assemblies. The blood having been drawn under oil with a minimum of trauma and transferred under oil into constricted tubes containing three drops of 20 per cent potassium oxalate (the reaction of which was repeatedly tested throughout the experiment), the determinations were made within fifteen minutes following all of the precautions recommended to insure accurate readings with our apparatus. The readings at room temperature were corrected after the method described by Myers and Muntwyler, *J. Biol. Chem.* **78**, 243 (1928). The Coleman pH electrometer was used, which has a standard error of ± 0.01 pH. I have here discussed the pH determinations because of the basic significance for all other biochemical changes. The other determinations revealed corresponding variations.

Were we simply satisfied with averaging pH levels for our three subjects, which for these determinations were:

$$H = 7.42$$

$$E = 7.41$$

$$S = 7.43$$

the entire impression would be faulty because this level is only approached one-fourth of the time.

The wide fluctuations are beyond the range currently accepted as normal; actually, a pH of 7.50 was exceeded in 14 days out of a possible 132. It becomes evident on inspecting the curves that these crests occur as fastigia in rather rhythmic fashion.³

With such wide swings the query immediately arises, are such observations reliable? Do these wide amplitudes really represent biological swings or are we dealing with technical faults (4)?

The simplest demonstration of the validity of these changes can be made if we merely determine the pH of the nasal mucous membrane observed at the time when the blood was drawn.

Nasal pH

pH determinations were made on the triplets at the same time each morning, glass electrodes being inserted into the nose directly on the mucous membrane and observations were made each minute for a twenty-minute period. The pH changes during the course of the readings and on some days this swing may be quite great. On the graph (Fig. 2) the vertical black lines indicate the maximal and minimal reading for each day. For the three men the correlation coefficients were again significant. When we now subtend the nasal pH under that of the blood pH for the subject H (Fig. 3), it is immediately apparent that we deal with a typical membrane effect—the trends are opposite. The correlation coefficient, while negative, is again significant. The actual range of the nasal pH is much greater than that of the blood, varying from a low of 5.00 (H) to a high of 7.90 (S).

How are we to account for such changes? Before proceeding to this question I shall briefly discuss related observations made on these subjects at the same time.

³ It would exceed the spatial limits of this paper to discuss at length the observations related to the problem of the change in pH levels here presented (i.e. the interrelation of lactic acid, basal metabolic rate, respiratory rate, etc.).

Other Observations

A wide variety of observations were made on the subjects and in Table I the correlation coefficients for the series are presented. Apart

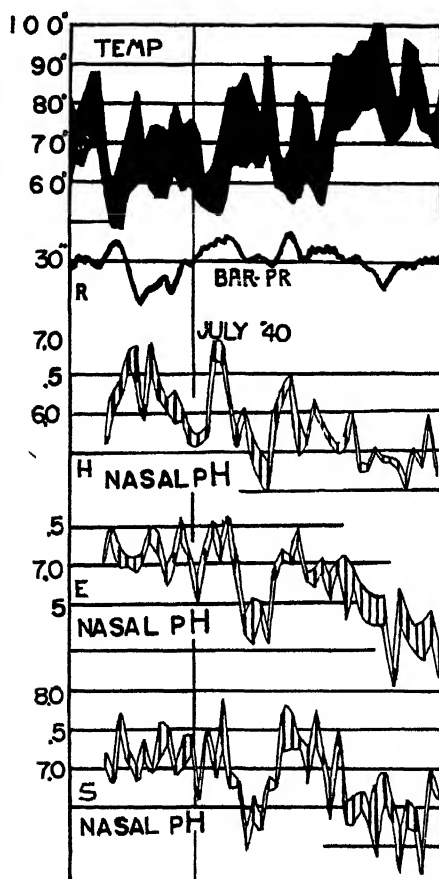


FIG. 2. Nasal pH Determinations of the Triplets
Meteorogram has been superimposed

from the blood chemistry the observations included: the blood cellular and physical changes; urine determinations; daily change in the volume of the arm and leg; the basal metabolic rate, weight, and breath holding time; muscular fatigue; and determinations of blood pressure, pulse

rate, body temperature, and respiratory rate.⁴ The actual range in variation is shown in Table IA.

Almost without exception the correlation coefficients were positive and, in most instances, highly significant, reaching a maximum for arm volume (.9).

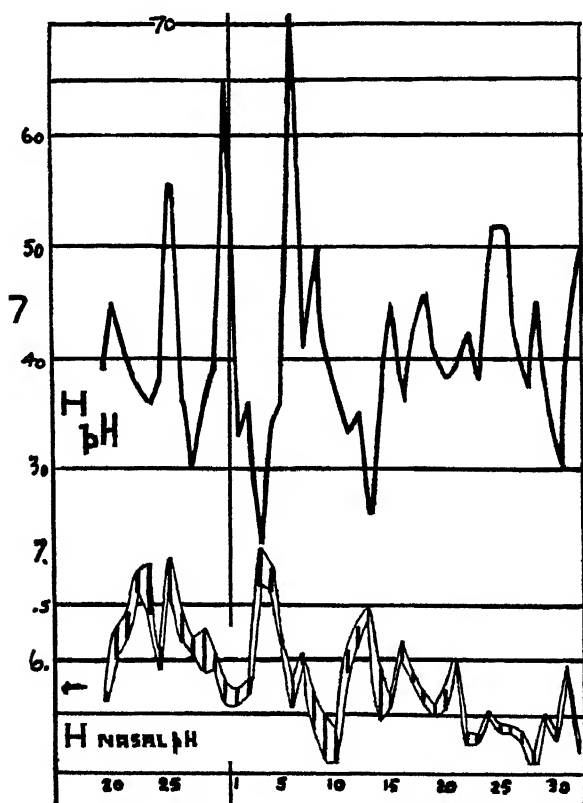


FIG. 3. Blood pH Curve Superimposed over Nasal pH Determinations of Subject H

It is only when we come to the observations in the cardio-vascular group that the correlations are not always significant. The reason for

⁴ In addition to these observations, the mental reactions of the subjects were tested in a variety of ways (addition test, *QRST* test, Rorschach test; the subjective state, clinical well being, etc.; the eye adaptation time, etc.).

TABLE I
Coefficients of Correlation Between Subjects

	No. of Observations	S:H	S:E	H:E
Arm volume.....	33	.875	.834	.900
Lactic acid.....	36	.777	.854	.847
Total fatigue.....	44	.568	.519	.796
R. B. C.....	42	.772	.663	.662
Seconds of breath holding.....	42	.619	.611	.771
Sedimentation rate.....	40	.560	.752	.406
Ionic calcium.....	44	.597	.750	.514
Urine pH.....	43	.623	.518	.738
Urine volume.....	43	.540	.356	.727
Creatinine.....	42	.709	.665	.481
Specific gravity.....	42	.452	.223	.699
Urea.....	33	.651	.687	.683
Blood pH.....	44	.680	.676	.670
Total nitrogen.....	33	.591	.512	.677
Uric acid.....	41	.516	.272	.654
Total acid output.....	43	.412	.357	.645
Weight.....	37	.522	.611	.425
Total solids.....	39	.602	.518	.342
Hemoglobin.....	43	.600	.563	.366
Protein.....	38	.430	.562	.448
B. M. R.....	28	.497	.552	.359
Cell volume.....	41	.260	.529	.361
Body temperature—afternoon.....	38	.428	.405	.522
Total dynamometer.....	43	.505	.487	.434
Pulse rate—afternoon.....	39	.497	.501	.209
CO ₂	41	.448	.420	.479
Total ammonia.....	43	.217	.331	.462
Leg volume.....	43	.318	.233	.398
Body temperature—morning.....	42	.281	.056	.337
Pulse rate—morning.....	41	.327	.028	— .071
Coagulation time.....	39	.304	.044	.155
W. B. C.....	42	.287	.203	.248
Systolic blood pressure.....	44	.141	.125	.266
Respiratory rate—afternoon.....	37	.128	.198	— .050
Diastolic blood pressure.....	44	.125	.128	.124
Respiratory rate—morning.....	40	.047	— .047	— .145

this resides in the fact that the lability of the cardio-vascular mechanism is so great that single observations are greatly influenced by transiently effective environmental factors. When observations are made more fre-

TABLE IA
Maximal and Minimal Values Observed in the Triplet Group

Blood	
pH.....	7.23-7.72
CO ₂ content.....	42-73 volume per cent
Lactic acid.....	5-25 mg. per 100 cc.
Ionic calcium.....	4-25 mg. per 100 cc.
Nasal pH.....	
	5.15-7.70
Urine	
24 Hour volume.....	630-2100 cc.
Specific gravity.....	1.011-1.029
Total nitrogen.....	10.22-21.80 g.
Urea nitrogen.....	10.5-18.0 g.
Ammonia.....	.108-.850 g.
Uric acid.....	.30-.78 g.
Creatinine.....	1.04-2.91 g.
pH.....	4.95-6.35
Acid output.....	158-630 cc. .1 N NaOH
Hemoglobin.....	
	12.5-19.0 g. per 100 cc.
Red blood cells.....	3,950-6,400 (000) per cc.
Cell volume.....	42-58 per 100 cc. blood
Leucocytes.....	4,100-8,300 per cc.
% PMN.....	33-73
Eosinophiles.....	.3-7.3
Sedimentation rate.....	1.5-11.5 mm. in hour
Coagulation time.....	$\frac{1}{2}$ -1 $\frac{1}{2}$ minutes
Total protein.....	6.8-9.2%
Total solids.....	15-27 mg. per cc.
Basal metabolic rate.....	+1- -22%
Maximum weight variation.....	3.9 kilo
Maximum variation in arm volume.....	8.5%
Maximum variation in leg volume.....	8%
Fatigue.....	15-72 seconds holding time
Breath holding.....	33-124 seconds
Blood pressure	
Systolic.....	104-130
Diastolic.....	64-84
Temperature—afternoon.....	98°-99.6°F.
Respiratory rate.....	12-28
Pulse rate.....	54-104

quently, the concordance becomes significant—as it does, too, when patients are kept at rest in bed.

The conclusion is permissible that during the six-week period of observation the reactions of the three like individuals to the environmental situation in which they existed were identical.

PART II

The environmental situation of these three young men was identical and, apart from the weather, practically uniform for the six-week period as far as exercise, hours of rest, etc. were concerned; there were no undue mental or emotional stresses; the food intake was identical. The major changes in the pH levels that have been presented must therefore, in

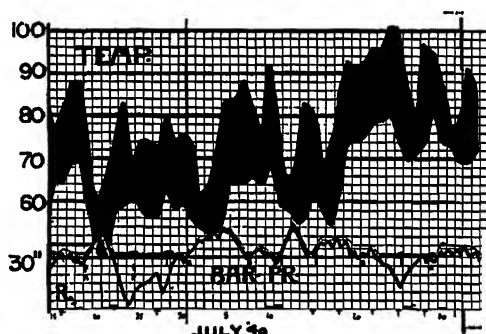


FIG. 4. Meteorogram for the Period of Observation

Maximal and minimal temperatures are indicated in the upper black field, barometric pressure in the lower trace, and rainfall is indicated above the date line.

some fashion, be related to the meteorological environment, that being the only common factor that changed greatly during the time of study.

The Weather of the Time

It is not my purpose to present an air mass analysis for the period under consideration⁵—the meteorogram (Fig. 4) will indicate the change in temperature and pressure. The upper curve is the daily maximal and minimal temperature, the lower curve is that of the barometric pressure. It will be noted that a series of "cold waves" passed over the region, with crests on the 20th and 21st of June (the coldest observed June weather for many years). Early in July and in the middle of the

⁵ In the detailed publication of this extensive material, a complete air mass analysis will be provided.

month there followed undue heat (temperatures to 102°F.), and again there was a final cold wave (relative) at the end of the period of observation. Precipitation occurred on June 21-22, again during July 9-10-11, and toward the end of the month. On other days there was slight precipitation.

Urine Examination

To make it immediately evident that these major changes in the weather alter the body chemistry, the correlation coefficients for the urinary determinations (using the means of the three subjects), and the maximal and minimal temperatures and barometric pressures are presented in Table II.⁶

TABLE II

Correlations with Maximum Temperature, Minimum Temperature and Barometric Pressure

	Maximum Temperature	Minimum Temperature	Barometric Pressure
Urine volume	-.5581	-.4866	.6946
Specific gravity1613	.3218	-.3553
Urine pH	-.3991	-.4298	.2076
Total acid3469	.3883	.0700
Total nitrogen0834	-.2068	.3562
Urea	-.3423	-.3330	.3942
Total ammonia	-.1486	-.0599	.1498
Creatinine	-.2941	-.2202	.1067
Uric acid1353	.2140	.2925

In Table III the interrelationship of the various urinary constituents is made evident in the statistically significant correlation coefficients.

The Ammonia Excretion

Because of its general importance I present the curve of the ammonia excretion of Subject H for the purpose of graphic orientation (Fig. 5). Each period of cold (with its relative anoxia) is reflected in an increase

⁶ I would again stress that for purposes of statistical expediency and accuracy we have here used but the common weather indices, *not* air mass analysis. Even so, statistically significant relationships become evident. In many instances lagging the relationship greatly improves the significance of the correlation coefficients. While significance is reached at .3, it should be noted that this material is not random but in sequence, in itself of importance in making the results more certain.

TABLE III

Correlations between the Various Urine Analyses Which Were Made

18 Urine vol- ume	19. Urea	20 Total acid	21. Urine pH	22 Total nitro- gen	23 Total am- monia	24 Cre- atinine	25 Uric acid	26 Specific gravity	
	+648	+032	+381	-052	+156	+296	+420	-816	18. Urine volume
		+359	+129	+026	+104	+248	+193	-369	19 Urea
			-643	-198	+550	+063	+217	-074	20 Total acid
				+230	-637	+305	+068	-170	21. Urine pH
					-197	+062	-276	-045	22 Total nitrogen
						-195	+059	-210	23 Total ammonia
							+261	-052	24 Creatinine
								-004	25. Uric acid
									26 Specific gravity

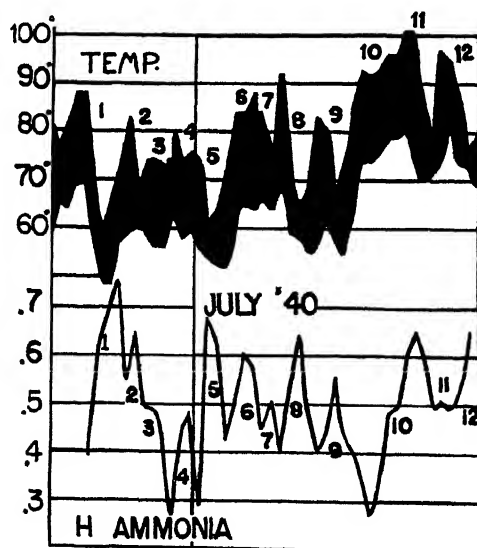


FIG. 5. Meteorogram to Illustrate Variations in the Ammonia Excretion as Related to the Weather

The lower curve indicates the daily excretion of ammonia. The upper curve indicates maximal and minimal temperature (in the black field). Periods of increase in the output of ammonia are numbered 1-12 and correspond to periods when environmental temperatures were decreasing.

in the ammonia output. An interference in this relationship occurs with the period of great heat (episode 11), and ammonia levels increase at a time when an alkalotic crest is evident (on the 24th and 25th).

The curve makes evident the wide pendulation established by the changing air mass.

The pH and Weather

If the urinary constituents change with the weather in a manner that is statistically significant when we merely use such crude indices as

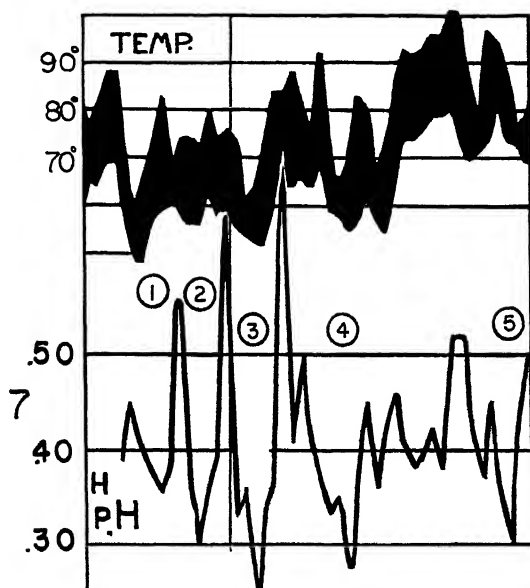


FIG. 6. The pH Curve of Subject H is Subtended Below the Environmental Temperature Curve of the Time

Periods of low pH (1-5) levels follow cold

temperature or barometric pressure and related to 24-hour samples (which actually should, in many instances, be lagged for statistical analysis), it will be worthwhile to return to a consideration of the pH curves and to study some of the interrelations that can be observed by direct inspection of the day-by-day curves. •

In Fig. 6 the pH curve of Subject H has been subtended below a curve of maximal and minimal daily temperatures for the period. Five

periods of low pH levels are indicated by circled numbers 1-5 (June 23 and 27, July 3, 13, and 30), with progression to the third episode. When we examine the curve, it becomes evident that these follow periods of lower temperature—the first occurring two days after the crest of the cold wave (this was the coldest period in June for many years); the second, on the day of the low; the third and fourth, on the crest of the cold wave; the fifth, representing a summation episode following two periods of cold, actually coincides with the sharp decline of the second period.

Diastolic Blood Pressure

We next select the blood pressure curve of Subject H in order to relate it to the weather of the time (Fig. 7).

Periods of increased diastolic blood pressure occurred in the following episodal fashion:

Episodes 1-2.....	June 21, 22, 23
3-4.....	June 25, 26, 28
5-6.....	July 1, 3
7.....	July 6
8.....	July 8, 9
9.....	July 12, 13
10-11.....	July 16, 17, 18
12.....	July 22
13.....	July 25
14.....	July 29, 30

We observe that periods of higher systolic blood pressure (June 22, July 2, July 17) occurred with the major cold waves evident in the meteorogram.

We also note that the numbered diastolic pressure episodes relate in general to periods of cold although there are exceptions (episodes 2, 7, 12) related to an undue sympathicotonia from some other cause; all three are related to rapidly rising environmental temperatures—12 at a time when a minor barometric crest was observed.

Periods of increasing diastolic blood pressure are significant of general increase in arteriolar tone, with related peripheral vasoconstriction and consequently, with a state of anoxymbiosis. Under such conditions we may anticipate the phase to be followed by a lowering of pH levels, because of the production of more acid metabolites when oxidation proceeds under conditions of relative anoxia.

In the next figure (Fig. 8) the pressor curve of Subject H has been extended over the pH curves and the numbered episodes of the diastolic increase (1-14, noted in Fig. 7) are indicated over the pH curves of the three individuals.

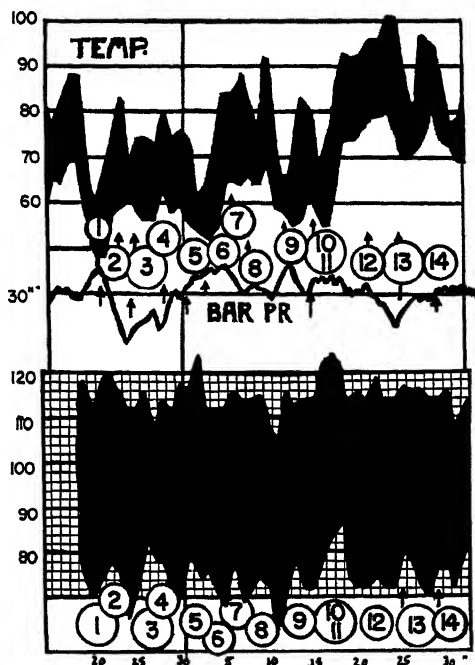


FIG. 7. Blood Pressure Curve Subtended under Meteorogram of the Time

Periods of increase in diastolic blood pressure are numbered 1-14. They correspond to periods of passing polar air. Lowest diastolic pressure (July 11) is associated with high temperature.

The individual changes in blood pressure levels are reflected in the pH curve, although to different degrees in the pattern of the three subjects.

In Subject H, for instance, the episodes 1-2 are fused; in Subject E, are separate; in Subject S, episode 1 is broader and 2, merely extended for one day. The episodes 7-8-9 are reflected in Subject H in a three-stage decline; in E, the lowest pH level is reached two days sooner, making episode 10 broader; in Subject S, episodes 7-8 are closely related, 9 appears as a separate episode, and 10 is exaggerated.

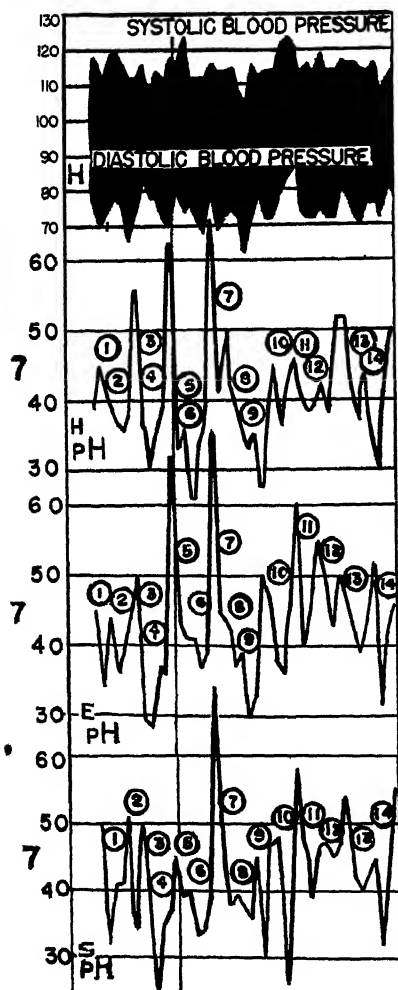


FIG. 8. The Blood pH and the Blood Pressure

The numbered episodes of Fig. 7 correspond to periods of decreasing pH in all three subjects.

My associates Berg and Mayne have established the statistical validity of the association of weather and the leucocyte count (5).

A study of these curves makes it apparent that the pH levels (even though observed but once a day!) present a clear-cut reflection of the changes in the weather.

The Leucocytes

If the pH levels of the blood reflect weather change, if the mechanism of this change is associated with change in vascularization, then we have the right to infer that every other biochemical level is also influenced—that the total organism is conditioned by the changing air mass.

It is not my purpose to demonstrate this by further chemical integration. I turn for a final objective to the leucocyte count, the level reflecting a series of factors: production, release from the leucopoietic tissues, rate of destruction, balance of distribution between splanchnic and peripheral vessel beds, etc.

In Subject H the count varied from a high of approximately 7500 to a low just under 5000. The graph is subtended under the pH curve and lagged one day (Fig. 9).

We note that the low phases of the pH curves (numbered 1–15) are met by an immediate increase in leucocytes—only with episode 5 is this missing, and here we probably witness either a fatigue phenomenon or one of negation.

I have so far taken it for granted that this meteorobiochemical integration involves chiefly temperature changes. But I would like to emphasize that with changing air mass we deal not alone with air temperature—pressure, wind velocity, humidity all change with the air

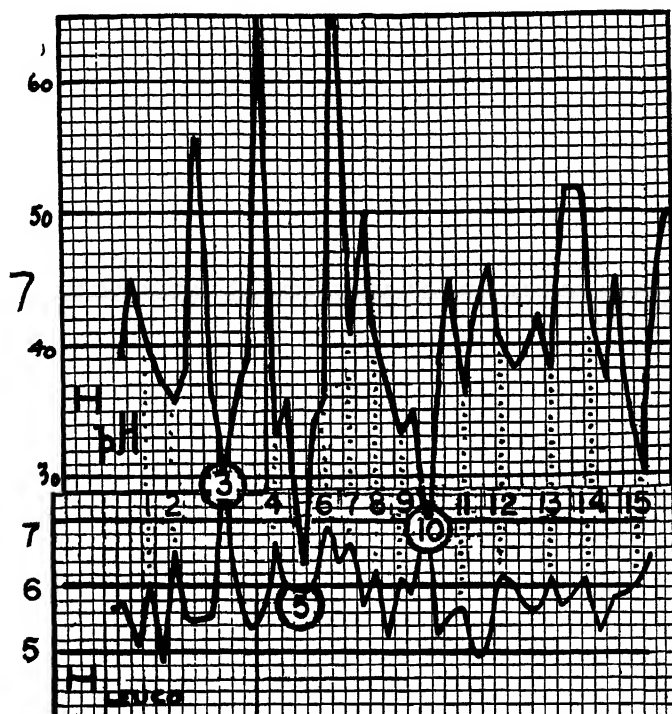


FIG. 9. Leucocyte Curve of Subject H

The pH curve of the same subject is extended above to illustrate the increase in leucocytes with low phases of pH. The leucocyte curve has been lagged one day, and the numbers at the left represent thousands.

mass; a series of other forces may be effective—the transmission of light rays, the ionization, the potential charge, minor waves in the air pressure (variometric determinations), the periodicity and amplitude of the changes; and, never to be forgotten—the state of the body when the effect is experienced. The reaction depends on the condition of the individual parts of the body and the body as a whole.

PART III

The Patho-Physiological Significance of the Biochemical Tide

A consideration of biological pendulation and the obvious summation phenomena in the chemical changes (for instance, the high pH levels of June 25, 30, July 6) must carry with it the implication that such wide deviations from the norm may, in some individuals, exceed the possibility of adequate equilibration, either for the organism as a whole or for certain organs or tissues. In other words, that dysfunction may result, that symptoms may become evident when the amplitude of the wave is too great.

A. Smooth Muscle Spasm. Smooth muscle and connective tissue reveal an increase in tone with relative alkalosis; consequently, blood pressures may increase. We might anticipate hemorrhage to occur in certain individuals with defective blood vessel walls. In other individuals who have scarred or irritable sphincters (peptic ulcer, biliary ducts, ureters, etc.) we might anticipate obstructive symptoms to appear or to be accentuated.

B. Tissue Hydration. In Fig. 10 change in the arm volume of the subjects is indicated. A fastigium was reached late in July. It must be obvious that if a patient has some inflammatory lesion located in a region where surrounding structures are relatively rigid, the accentuation of sudden tissue swelling will result in localized pain and, if the pressure continues, may cause tissue necrosis when swelling and the consequent anoxia of sufficient degree is established. This is the characteristic picture that we witness in the alveolar abscess (toothache) or in osteomyelitis. It underlies arthritic pain as well as a great variety of other clinical symptoms.

C. Thrombosis. If a phase obtains when, with falling diastolic blood pressure and with lowered pH levels, the coagulation time is markedly shortened (as it was in Subject H on July 12), the trend to thrombosis would be materially enhanced and in a susceptible tissue area (traumatized, infected, or anoxic) actual clotting may take place.

D. Diabetic Coma. If a phase obtains in a diabetic when peripheral vessels contract (sympathicotonia) and more sugar is released from the liver, the while less is burned peripherally, blood sugar will rise. If, at the same time, acid products of anoxia accumulate and the pH falls, if now tissues begin to swell (the brain included)—we have the background for a diabetic coma.

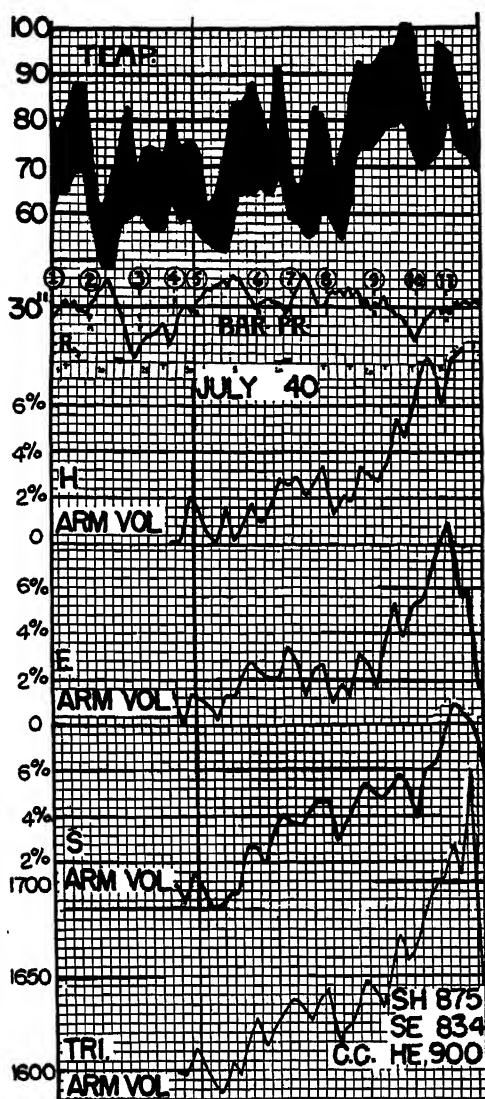


FIG. 10. The Fluctuating Tide of Arm Volume (Hydration, increase in Vascularity, etc) in the Triplets

An average of the actual change in volume for the three men is shown in the lower curve.

E. Resistance to Infection. If a phase obtains at a time of falling diastolic blood pressure, of low pH, of increasing hydration, and shortened coagulation time, and if at the same time the proportion of polymorphonuclear leucocytes to lymphocytes is maximally lowered, we might anticipate that resistance to the penetration of bacteria or resistance to infection in general might be lessened.

A number of theoretical as well as very practical implications follow in the wake of these considerations.

First, the meteorological environment provides situations that are never repeated as far as the individual organism is concerned. It is true that during the period under consideration maximum temperatures were 80°F. on June 28 and 80°F. on July 27, and statistically such days might be regarded as identical, actually air pressure was *low* on the 28th of June and *high* on the 27th of July. In this instance the 80° on the 28th of June reflected the passage of a tropical air mass, while, in the other instance, the day with maximal temperature of 80° was associated with the passage of a cold air mass.

Most important is the fact that on the 28th of June the subjects had been living for five days with maximal temperatures ranging from 70° to 82°F. while on the 27th of July the preceding five days had registered temperatures from 90° to 102°F.; obviously the subjects were conditioned by the *preceding* weather.

Second, the reaction of the individual will depend on the frequency of the passage of the air masses. As with simpler phenomena in the physics of wave motion, the effect on the human may cause summation as well as negation. In the pH curve in particular, the evidence of amplification is clearly evident in the rising pH crests.

Third, the effectiveness of the biochemical pendulation to the extent of dysequilibration (i.e., the precipitation of symptoms of disease, or death) will of necessity depend on the base line. In the late winter we are relatively more acid because of the loss of buffers and all the other organic and inorganic reserves. Then major wave motion will result in periods of unusually low pH levels with consequent pathophysiological effects. In the late summer and autumn this situation is reversed.

Fourth, while it has been demonstrated that these triplets reacted in an identical fashion, it must be obvious that dissimilar individuals will react differently in the amplitude, acuity, and even direction of response to environmental stimuli such as those provided by the air mass, de-

pending on as many factors as underlie dissimilarity of the individuals in the population.⁷

The demonstration of the effectiveness of air mass change on the organism carries with it the implication that it is a factor that must be evaluated in every clinical or experimental observation. Actually, this should not prove a handicap to the investigator, for this inclusion in the interpretation of the results will not infrequently explain puzzling paradoxes and inconstant results.

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⁷ Day-by-day observations on dissimilar individuals have been published in a series of monographs dealing with normal and sick individuals, "The Patient and the Weather" (3).

On the Mechanism of Enzyme Action. Part 20

Chemistry of Dehydrogenations with and without Training of *Fusarium lini* Bolley (FIB)*

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INTRODUCTION

A number of molds are known which possess the capacity to convert sugar into alcohol. Of these a representative group includes various strains of *Aspergillus*, *Mucor*, and *Citromyces*. If precautions are not taken to control the amount of oxygen available, the alcohol so formed in the sugar decomposition is rapidly converted to various other endproducts, usually fairly complex acids. Large quantities of fumaric, succinic, oxalic, and citric acids, among others, are obtained in this way (1, 2, 3). FIB., on the other hand, more closely resembles (4) yeast than the other molds in its activity. The alcohol produced is not attacked so rapidly and only small quantities of the organic acids mentioned above are obtained.

This study represents an extension of those reported (5) concerning the action of FIB. on various homologues of methyl alcohol and other hydroxylated compounds. Within the past both bacteria and molds have been employed to investigate the changes brought about to the various substrates used here. Data obtained has been gathered together (3) and discussions can be surveyed there.

METHODS

The basic inorganic constituents of the nutrient media were supplied by the following substances. All chemicals were of tested purity grade and their concentrations are expressed in grams per liter: potassium

* This study was aided in part by a grant from the Rockefeller Foundation. The authors' appreciation is extended also to the Atlas Powder Company, Wilmington, Del., for the supply of the various hexitols used in this investigation.

nitrate 5 g.; mono-potassium phosphate 4 g.; magnesium sulphate + 7H₂O 0.75 g.

The general procedure as outlined (5), was followed to establish the qualitative course of the degradation of the organic substrates used as sole carbon sources for this micro-organism. When the fixation method was employed to trap aldehydic dissimilation products, one gram of this reagent was dissolved in each liter of nutrient media prior to sterilization and inoculation.

In experiments where mycelium weights were to be determined, the inoculated flasks were left undisturbed in the incubator (6a) until the time of analysis. At that time, the mycelium was filtered through tared porous alundum crucibles and washed with four successive 25 ml. portions of distilled water. The washed mycelium and crucible were thereupon dried overnight at 60°C. and weighed. The weight of the dried mycelium was obtained from the difference between the initial and final weights. All reported values are the average of at least duplicate determinations.

Utilization of glucose by FIB. was followed by means of the Shaffer-Somogyi (6) method. This analytical procedure was used to determine the reducing substances present in various other dissimilations. In order to apply this method to the compounds qualitatively shown to be present, known solutions were analyzed where such were available. In addition, the methods of Kruisheer (7) and Englis and Byer (8) were applied.

Acetyl methyl carbinol was detected in the culture media by means of the formation of nickel dimethylglyoxime (9) after oxidation with ferric chloride. Analyses carried out without previous treatment with ferric chloride gave negative results thus indicating the absence of diacetyl. It was identified by means of p-nitrophenylhydrazine and quantitatively analyzed for by the method of Werkman et al. (10).

The Lu reaction (11) was carried out to detect pyruvic acid and isolation of the 2,4-dinitrophenylhydrazone of this substance served to establish its presence. Ethyl alcohol was tested for in the volatile neutral distillate by means of the iodoform (12) and dichromate-nitric acid (13) reactions.

Phenylhydrazine was used to isolate derivatives of the reducing compounds formed from erythritol and sorbitol culture media and the method suggested by Shriner and Fuson (14) was employed.

EXPERIMENTAL¹

The qualitative course of the degradation of the substrates is given in Table 1. An examination of the table indicates that, in general,

TABLE 1
Qualitative Analysis of Substrates

Substrate	Dissimilation Product	Method	Identification	Analysis*	
				Calc. Found	Calc. Found
Methyl alcohol	Formaldehyde	Trapping	Dimedon deriv. m.p. 186-187° (aq. alcohol)	C 69.86, 69.71	H 8.21, 8.16
Ethyl alcohol	Acetaldehyde	Trapping	Dimedon deriv. m.p. 140-141° (aq. alcohol)	" 70.60, 70.84	" 8.50, 8.40
Ethylene glycol	Glycolaldehyde	Trapping	Dimedon deriv. m.p. 224-226° (aq. alcohol)	" 71.10, 71.09	" 7.90, 7.96
2,3 Butylene glycol	Acetyl methyl carbinol	Isolation	p-Nitrophenylosazone m.p. 318° (pyridine-acetic acid)	N 23.60, 23.54	
Glycerol	Triose	Isolation	2,4 Dinitrophenylosazone m.p. 264-265° (pyridine-acetic acid)	" 25.00, 25.04	
	Pyruvic acid	Isolation	2,4 Dinitrophenylhydrazonem.p. 214-215° (acetic acid)	" 20.89, 20.91	
	Ethyl alcohol	Isolation	Iodoform & dichromate-nitric acid test		
Erythritol	Erythrulose	Isolation	Phenylosazone m.p. 163° (aq. alcohol)	" 18.79, 18.87	
Glucose	Acetaldehyde	Trapping	Dimedon deriv. m.p. 140-142° (aq. alcohol)	C 64.43, 64.58	H 6.04, 6.22
Sorbitol	Hexose mixt.	Isolation	Phenylosazone m.p. 163-164° (aq. acetone)	" 70.59, 70.46	" 8.50, 8.51
Mannitol	Pyruvic acid	Isolation	Lu test	" 60.34, 60.32	" 6.14, 6.35
Dulcitol	Pyruvic acid	Isolation	Lu test	N 15.64, 15.73	
Inositol	No dissimilation products detected				
Tertiary butyl alcohol	No growth observable				

* The authors' appreciation is extended to Mr. J. Alicino for these analyses.

the manner of utilization of these compounds parallels those investigated previously.

As a result of the study of the dissimilation of n-propyl, iso-propyl,

¹ Abridged from part of a thesis submitted by G. J. G. to the Graduate School of Fordham University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1942.

n-butyl, sec-butyl alcohols and propylene glycol, it had been observed that their dehydrogenation followed definite paths depending upon the alcoholic group present in the substrate molecule. When the alcohols investigated contained but secondary alcoholic groupings, there was marked accumulation of the intermediate products, while the contrary was true of the primary alcohols. In addition, a compound containing

TABLE 2
Yields of Dimedon Derivatives Obtained by Trapping

Substrate	Percentage composition	Dissimilation product	Precipitate weight*
			g.
Methyl alcohol.....	0.2-0.4	Formaldehyde	0.03
Ethyl alcohol.....	0.4	Acetaldehyde	1.00
Ethylene glycol†.....	2.0	Glycolaldehyde	0.15
Glucose.....	0.5	Acetaldehyde	0.10
Erythritol-glucose.....	1.0	Acetaldehyde	0.12

* Quantity obtained from 10 flasks each containing 100 ml. nutrient media.

† Due to inhibition by dimedon this experiment had to be carried out over a period of 12-15 weeks.

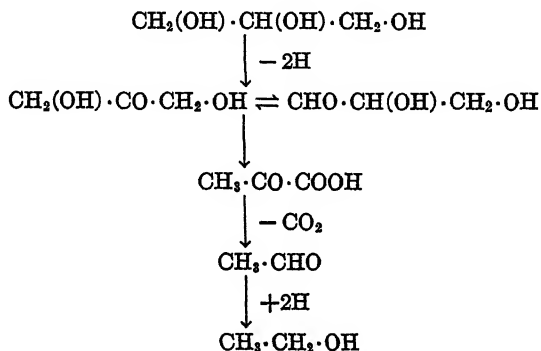
TABLE 3
Maximum Yields of Dehydrogenation Products Observed

Substrate	Percentage composition	Dissimilation product	Percentage yield
2,3-Butylene glycol.....	0.4	Acetyl methyl carbinol	25-30
Glycerol.....	2.0	Triose	0.3-0.5
Erythritol.....	1.0	Erythrulose	3-4
Sorbitol.....	4.0	Hexose mixture	3-4

a mixture of the two groups when acted upon by FIB., resembled a secondary alcohol in this respect.

In this work no accumulation of intermediates was observed with either ethyl alcohol or ethylene glycol. Rather, the substances shown to be present had to be trapped (Table 2) in order to be determined. When 2,3-butylene glycol, glycerol, and erythritol were used as carbon sources not only isolation of the primary (Table 3) dehydrogenation products was possible without trapping but also compounds other than these were detectable in the glycerol experiments.

One path of the glycerol dissimilation appears to be *via* dihydroxyacetone, pyruvic acid and ethyl alcohol since small amounts of the same could be isolated, i.e., 175 mg. and 350 mg. of the 2,4-dinitrophenylhydrazine derivatives of the trioses and pyruvic acid respectively along with approximately 75 mg. of alcohol from one liter of a two per cent solution of glycerol nutrient media. This sequence of reactions is one which may take place also in the latter phases of the sugar degradation in alcoholic fermentation. The glycerol serves as a hydrogen donator for the reduction of the acetaldehyde obtained after the decarboxylation of pyruvic acid:



Gould (15) has indicated that the enzymes of *Fusaria* causing alcoholic fermentation are "constitutive" regardless of the substrate upon which they are grown and, that the amount present varies with the compound employed.

The utilization of hexitols by FIB. gave rise to variable results. When dulcitol and mannitol were used as sole carbon sources, only pyruvic acid, as shown by positive Lu tests, could be established as a degradation product. Neither reducing compounds nor ethyl alcohol could ever be detected. Inositol proved totally negative in respect to the analyses for all the compounds just mentioned. Sorbitol yielded a hexose mixture, one of the constituents which was sorbose along with other undeterminable compounds. The presence of sorbose was established by positive Seliwanoff tests (16) and phenylsorboseazone formation both before and after hypiodite removal of the aldoses present.

Experiments were carried out using an oxygen atmosphere as well as aeration and passage of oxygen through the media in order to obtain

greater yields of the dehydrogenation products of glycerol and sorbitol. Beyond an initial acceleration of this process, the results indicated that such treatment merely enhances the assimilatory processes without increasing the percentage yield of reducing substance.

Preliminary experiments using methyl alcohol and erythritol as sole carbon sources were in agreement with the observations of Nord (17) and Nedig (18). No growth could be observed in solutions having concentrations of methyl alcohol as low as 0.2%. In the erythritol experiments germination of FIB. but no mycelium formation was observed. It was, therefore, decided to use glucose as a growth substance along with these unattackable substances in a series of mixed fermentation experiments.

The dimedon fixation method was employed in the experiments with methyl alcohol and 0.01 ml. of this substrate was added one week after inoculation to a 0.5% glucose fermentation. This addition was continued weekly for a period of three to four weeks and resulted in isolation of small quantities (approx. 20–30 mg.) of the dimedon derivative of formaldehyde from 10 culture flasks each containing 50 ml. of media.

As controls in these experiments a series of sterile uninoculated glucose dimedon media was treated in a similar manner and no precipitate was observed. In addition, 0.5% glucose-dimedon medium was inoculated and the derivative of acetaldehyde was obtained after the fermentation had proceeded one week. At the time the precipitation was first noted, the glucose initially present had practically disappeared and there were only traces of pyruvic acid present. Hence, the formation of acetaldehyde is due to a dehydrogenation of the alcohol formed in the fermentation of glucose. As is known, this compound is an intermediate in the yeast fermentation of hexoses also, but under the experimental conditions used here no trapping of the intermediate of this process was evidenced since the dimedon derivative isolated appeared only after the glucose initially present had been removed. This derivative disappears over a period of three to four weeks.

In erythritol experiments 1% solutions of this substrate along with 0.5% glucose was prepared with the usual inorganic nutrient medium. This solution was then measured into flasks, sterilized, inoculated and periodically analyzed for reducing compound present and mycelium weight. Fig. 1 represents the results obtained.

The reduction value in the control flasks containing glucose alone ultimately dropped to zero and remained there whereas that of the mixed fermentation fell almost to zero and then subsequently rose again. Mycelium weights in the glucose controls increased to a maxi-

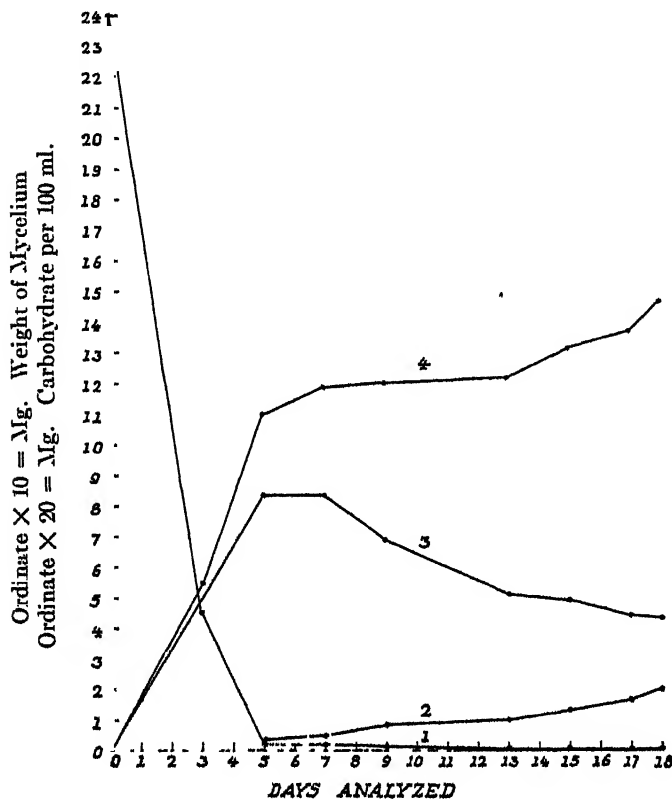


FIG. 1. Dehydrogenation of erythritol. 1. Glucose reduction. 2. Glucose-erythritol reduction. 3. Glucose mycelium weight. 4. Glucose-erythritol mycelium weight.

mum at that time when the glucose present had been reduced to a minimum, remained there for a period of two days and then decreased sharply. This latter effect is in all probability occasioned by the fungal utilization of its reserve materials. In the mixed fermentation of glucose and erythritol the mycelium weights continued to rise, thus indicating an assimilation of erythritol.

In order to establish the identity of the reducing compound formed, the phenylosazone was prepared. This corresponded to that of a tetrose, being formed from either erythrose or erythrulose. To further establish the identity of the compound present, the following tests were carried out (Table 4).

The positive Seliwanoff reaction and formation of the phenylosazone of a tetrose both before and after hypiodite oxidation clearly indicate

TABLE 4
Qualitative Analysis of Erythritol-Glucose Media
Part A

Method of Analysis	Substance found	Identification	Analysis					
			C		H		N	
			Calc.	Found	Calc.	Found	Calc.	Found
Before hypiodite removal of reducing compounds								
Phenylhy- drazine Seliwanoff	Tetrose	Phenylosazone m.p. 163-164° (aq. alcohol)	64.43	64.58	6.04	6.22	18.79	18.87
	Ketose							
After hypiodite removal of reducing compounds								
Phenylhy- drazine Seliwanoff	Tetrose	Phenylosazone m.p. 163-164° (aq. alcohol)	64.43	64.59	6.04	6.04	18.79	18.63
	Ketose							

Part B

Analysis	Method	mg./5 ml. Concentrate
Total reducing	Shaffer-Somogyi	1.5
Aldose present	Hypiodite	0.1
Total reducing after hypiodite	Kruisheer	3.6

the presence of a ketose. In addition the total reducing value and that of the aldose present, along with analysis by the Kruisheer method, show that erythrulose is present in the mixture to the extent of about 80-85%.

A number of explanations can be offered as to the effective role of glucose in bringing about the changes observed when both methyl alcohol and erythritol were used as carbon sources. The most probable

is that this substance furnishes the means to produce a large quantity of this organism. As a necessary consequence the number of mutants probably present is increased and they can then utilize the compound previously considered unattackable. This hypothesis appears to be substantiated by the marked increase in the mycelium weights over that of the glucose controls observed in the experiments with erythritol-glucose mixtures. With respect to this it should be noted that Braun and Cahn-Bronner (19) utilized bulk inoculations to bring about the same effect in the case of *B. typhosum* requirement for tryptophane. The effects observed can be ascribed to a training² brought about by means of a special technique.

In order to evaluate the effect of dimedon on the growth and metabolism of FIB., the fixation method using 1 g. of dimedon per liter was applied to the compounds in this investigation as well as in the previous one. Mycelium weights were determined in both controls and in trapping experiments. In Table 5 are recorded these values, the extent of inhibition observed and the final pH of the controls.

Dimedon appears to exert variable effects upon the organism in the presence of the compounds used. The inhibition of growth is slight with hexitols, glycerol, and 2,3-butylene glycol which incidently do not give rise to trappable compounds. Duleitol and n-propyl alcohol utilization is inhibited completely in the presence of this reagent, no growth being observed after six weeks. Growth with ethyl alcohol which gives rise to a copious precipitate was inhibited approximately

² A training of FIB. had been evidenced also in the experiments reported in the previous paper (5b).

Note added August 26, 1942. During her tenure of the William J. Fordrum Scholarship, Rita C. O'Connor investigated certain phases of the breakdown of glucose, lactose and cellobiose. In a large series of experiments, cultures of FIB. with the first two carbohydrates were kept in flasks both in the presence and absence of oxygen or methyleneblue (in nitrogen). At the end of each experiment lasting several weeks, the flasks containing glucose in the absence of oxygen showed a remarkably greater accumulation of ethyl alcohol than did those with a hydrogen acceptor. In neither case did lactose give rise to detectable amounts of ethyl alcohol. Accordingly, the lactose degradation seems to be subject to the action of an enzyme system which is at least partly at variance with that which causes alcoholic fermentation of glucose. This was also borne out by CO_2 and lactose determinations, which indicated, in agreement with earlier observations by Nord and Engel (l. c.), that this carbohydrate is more rapidly dissimilated in the complete absence of Phosphorus donors.

3. The growth with all the remaining monohydroxylated compounds along with the various glycols was not completely inhibited but was so slow over the experimental period (i.e., 2 weeks) as to yield mycelium weights less than 10 mg. Zeller (20), in relation to the action of carbonyl reagents as inhibitors of various enzymatic processes is of the opinion that possibly reaction between groupings of the enzymes and the reagent may be responsible for the effects observed. However, the variability of the inhibition of growth by dimedon in the

TABLE 5

Effect of Dimedon on the Growth of FIB.

Initial concentration, 0.4%; initial pH, 4.4; dimedon concentration, 1 g. per liter.

Substrate	Final pH control	Mat weight, mg, control	Mat weight, mg, dimedon	Percentage inhibition
n-Propyl alcohol	4.7	*	0	
iso-Propyl alcohol	5.5	28	*	Approx. 100
Ethyl alcohol	6.5	66	40	39
Butyl alcohol	5.8	30	*	Approx. 100
sec-Butyl alcohol	5.8	36	*	Approx. 100
Ethylene glycol	5.8	35	*	Approx. 100
Propylene glycol	6.3	65	*	Approx. 100
Glycerol	6.5	140	103	26
2,3-Butylene glycol	5.9	48	20	29
Sorbitol	5.7	38	26	32
Mannitol	6.5	83	78	6
Dulcitol	6.5	77	0	100
Inositol	6.3	51	45	12

* Indicates mycelium weights less than 10 mg.

experiments with FIB. requires definite knowledge of the actual constitution of the enzymes present before an adequate explanation can be offered.

Additional information can be obtained from the changes in the pH of the media. The initial pH was 4.4 and in all cases the final value in the controls indicated a shift to the alkaline side. This observation excludes any appreciable formation of organic acids as dissimilation products. The experimental findings in this respect, when ethyl alcohol was used as sole carbon source, are in sharp distinction to those

reported by Anderson (21), i.e., the final pH in his experiments being 2.97. This difference has its explanation in the nitrogen sources present among the inorganic constituents of the nutrient media rather than the formation of organic acids in the one case and not in the other. Anderson used ammonium nitrate whereas potassium nitrate was the source of nitrogen in this work. Luz (22) has shown that in glucose fermentations when ammonium nitrate was present the nitrate ion was preferentially used as long as glucose was available. However, as soon as the glucose disappeared, the ammonium ion served as a nitrogen source even though nitrate ion was present. Evidently, in the case of Anderson's experiments the pH reported was occasioned by the utilization of ammonium ions leading to the observed shift towards the acid side. In this work, since only the nitrate ion was available as nitrogen source, it alone could be utilized and as a result, the media become more alkaline.

DISCUSSION AND SUMMARY

An adequate approach to the solution of the mechanism of dehydrogenations catalyzed by FIB. has as its essential part an understanding of the enzyme system concerned. As a contribution to the solution of this problem, a study of chemical conversions effected by the various enzymes present was undertaken.

Evaluation of the course of the dehydrogenation with primary, secondary, and tertiary alcoholic groups in the molecule indicated that of the three, the first two groups of compounds could be dehydrogenated whereas the third could not. The failure of the tertiary alcohol (Table 1) to serve as a carbon source may be attributed to the concurrent necessity of a rupture of the molecule along with the dehydrogenation process. Certain differences in the dissimilation of the primary and secondary alcohols were discernable which were enough to make valid distinctions between the two.

When additional compounds containing more than one hydroxyl group and with longer carbon chains were investigated, the differences observed between the primary and secondary alcohols held with compounds having as many as four carbon atoms in the molecule. When hexitols were used as sole carbon sources, this differentiation disappeared.

A particularly interesting finding, helpful to the interpretation of studies of alcoholic fermentation, was the retarded blocking with dimedon of glycolaldehyde as dehydrogenation product of ethylene

glycol. This compound has been occasionally considered (23) as an intermediate in pentose fermentations and the trapping of small quantities was not experimentally shown up to the present.

It has been assumed (21, 24) that the alcohol values of *Fusaria* fermentations were low due to the fungal utilization of alcohol and that acetaldehyde was determinable as an intermediate in the fermentation of glucose to alcohol. Under the present experimental conditions, the formation of acetaldehyde was observed only after the glucose had virtually disappeared. Hence, the formation of this substance, as isolated by means of dimedon, was due to a dehydrogenation of the alcohol produced from the glucose and not (25) as an intermediate in the conversion of the hexose to alcohol.

The omnivorous nature of this organism rendered the evaluation of the stepwise degradation of the intermediates formed from the various substrates extraordinarily difficult if not impossible. Of the compounds investigated only a very small number were found to be unsuitable as carbon sources and, of these the application of a specialized technique resulted in the "training" of this organism to dehydrogenate both methyl alcohol and erythritol.

The functioning of enzymes producing effects comparable to those in alcoholic fermentation was evidenced when glycerol was used as sole carbon source but could not be established in the experiments with various hexitols.

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Growth of Bacteria in an Iron-Free Medium

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The need for further knowledge of trace element requirements of bacteria, and especially of an understanding of the functions of inorganic ions in cellular metabolism long has been appreciated, and has recently been commented on by leading bacterial nutritionists and physiologists: Stephenson (15), Knight (7), Koser and Saunders (8), Lipman (9), and Foster (5).

Chief among the obstacles in this field is the lack of a medium sufficiently free from a trace element to reveal marked deficiencies. In general, the methods used to remove iron from media have been inadequate. Some results have been obtained with yeasts and fungi, but deficiency studies with heterotrophic bacteria have not met with success. This is readily understood when it is realized that bacteria usually require less than one-tenth the amount of iron needed by yeasts and molds. The few methods of trace-removal available utilize the principles of recrystallization, precipitation or adsorption. Practically all workers in the field have found that recrystallization is not successful. Burk and Horner (2), and others have found that the best known methods of adsorption, such as those of Steinberg (14), Hopkins (6), Elvehjem (4), Bortels (1), and Roberg (13) are not adequate for removal of iron from a bacterial medium. Precipitation methods are not successful because the precipitations are not complete in a biological sense. A medium that is "chemically free" from iron may still contain the metal in concentration two or three orders of magnitude greater than that "biologically free" from the element.

Burk and Horner (2), after reviewing and testing all known methods of purification, came to the conclusion that the only method of producing a really iron-deficient medium for bacteria was to use ingredients extremely free from the element. This is probably a good method, but such materials are not easily obtainable.

In these studies preliminary experiments with the adsorption methods of Bortels (1), Elvehjem (4), and Steinberg (14) gave good deficiencies with *Aspergillus niger*, but bacterial growth tests with *Aerobacter indologenes* (Iowa State 23B) showed no deficiencies. The old mold purification method of Molisch (10), which has been used by Molliard (11), and Roberg (13) was then tried. The medium was first made iron-deficient for *Aspergillus niger* by the method of Steinberg (14), and was then further purified biologically by a limited growth of this mold. The results of this method were very satisfactory. When the medium was tested with *Aerobacter indologenes*, only a trace of growth appeared, and when iron was added normal growth occurred. The main disadvantage of this method was in the length of time required to prepare the medium, which was about one week.

A study of the more modern methods of iron analysis revealed, among other things, the very unique properties of 8-hydroxyquinoline (oxine) in forming water insoluble ferrous and ferric hydroxyquinolates. The following method yielded the most satisfactory iron-deficient bacterial medium thus far obtained, and this in a time as short as one hour.

METHOD OF PURIFICATION

The basal medium used in this work had the following composition, with only slight variation from time to time.

(1) Dextrose (Bacto or Pfanstiehl C.P.)	10 g.
(2) K_2HPO_4 (Analytical Reagent)	4 g.
(3) KH_2PO_4 (Analytical Reagent)	1 g.
(4) $(NH_4)_2SO_4$ (Analytical Reagent)	1 g.
(5) $MgSO_4 \cdot 7H_2O$ (Analytical Reagent)	... 0.5 ml. of a 20% sol.
(6) Salt solution*	0.1 ml.
Water	.. 1000 ml.

Ingredients 1, 2, 3 and 4 are dissolved in 150 ml. of glass-distilled water and the resulting solution is filtered through a Whatman No. 42 or similar filter paper into a glass-stoppered pyrex separatory funnel. The stopcock of the funnel is not greased, being wet only by the solution or distilled water. About 5 mg. of crystalline 8-hydroxyquinoline (Eastman) are dissolved in 1 ml. of chloroform which is then poured into the funnel, shaken vigorously and allowed to stand for five minutes.

* The salt solution contains $ZnSO_4 \cdot 7H_2O$ —44 mg; $CuSO_4 \cdot 5H_2O$ —40 mg., $MnSO_4 \cdot 4H_2O$ —41 mg., and KI 42 mg. in 100 ml. of solution. This is kept in a pyrex glass-stoppered bottle and made up frequently.

The solution turns yellowish, the color of oxine in aqueous solution. About 3 ml. of redistilled chloroform are now poured into the funnel which is shaken vigorously for a minute and then rotated about a half minute to cause the droplets of chloroform to coalesce and come to rest on the bottom. It will be noted that the chloroform is black if ferrous iron is present, or a dark green with ferric iron. The chloroform layer is drawn off and the solution is again washed twice as explained above with 3 ml. portions of chloroform. The whole extraction is repeated by adding 5 mg. portions of the solid oxine directly to the funnel, shaking vigorously into the chloroform saturated aqueous solution and washing out the iron complexes until they are no longer formed. Three extractions are usually enough to remove all of the iron. After the last portion of oxine is added the solution is washed 3 times with 3 ml. portions of chloroform and once with 5 ml., allowing the excess chloroform to settle out for about ten minutes. The solution is then free of the oxine reagent which is very soluble in chloroform. The purified solution is now carefully transferred to a scrupulously clean 2 liter Erlenmeyer flask and immediately covered with a clean 250 ml. beaker used as a cap. All solutions must be carefully protected from dust which is a significant contaminant in trace element work. The purified solution may be kept for several days in the icebox, or it may be diluted and used immediately. Keeping deficient media longer than a week is not recommended. When the solution is to be used it is first diluted almost to volume with tested triple-distilled water, the MgSO_4 and salt solution are added, and the final dilution made. Adding the accessory salts to the concentrated purified solution results in their precipitation as phosphates. The excess chloroform remaining in solution will be removed when the medium is autoclaved but it is our practice to remove it by heating and shaking before the dilution is made. Care must be taken not to heat the solution vigorously, otherwise caramelization of the sugar will result.

Batches of the purified medium have been calculated to contain between 0.0007 and 0.003 part of available iron per million parts of solution (0.7 to 3.0 micrograms per liter) by extrapolation of growth curves. It is hardly necessary to mention that the purified medium must be subjected to the least amount of handling to prevent recontamination.

WATER

All water used in the analysis, in the dilution of the media, and in the preparation of solutions to be added to the purified medium, was ob-

tained by triple distillation at a slow rate in a large all-pyrex still having a 20-inch column to prevent droplet carry-over. Samples of 100 ml. of the water were tested by the diphenylthiocarbazone (dithizone) method as described by Stout and Arnon (16). The dithizone method is probably the most sensitive test for the presence of heavy metals in water. It can detect the addition of less than one microgram of zinc, lead, copper and several other metals to a liter of metal-free water.

GLASSWARE

All glassware used in the preparation of the medium and in the growth experiments was of pyrex. When pyrex and fused quartz culture flasks were compared in growth experiments, there was no detectable difference. Glassware was cleaned by the following method which gave the best results. The vessels were well brushed with soap solution, rinsed in succession with distilled water, alcoholic potassium hydroxide, distilled water, aqua regia, and distilled water. They were then filled with glass-distilled water and autoclaved at 20 lb. pressure for 30 minutes. When the autoclaving was omitted the glassware contained enough adhering iron to affect growth experiments. All culture flasks were covered with the proper size of beakers or other glass covers made for the purpose. No cotton plugs, corks or rubber stoppers were used in any of the work. The cleanness of the glassware was checked by rinsing it with triple distilled water and testing the rinsings by the dithizone method.

RESULTS

Representative results to be expected from use of the medium will be described. The exact ranges of iron concentration necessary for optimal growth of several species of bacteria have been determined. In the case of the test organism, *Aerobacter indologenes*, the growth deficiency curve (Fig. 1) shows that growth is a function of the iron added between zero and 0.025 p.p.m. With *Pseudomonas aeruginosa*, which is known to have a more elaborate cytochrome system than that of *Aerobacter*, growth increases with increased iron concentration from zero to 0.090 p.p.m. This organism therefore appears to need almost four times the iron concentration required by *Aerobacter indologenes*. The iron requirements of *Klebsiella pneumoniae* appear to be practically identical with those of *Aerobacter indologenes*. When the iron content of the medium is increased beyond the above figures there is no change

in the amount of growth until approximately three to four parts per million are reached. Precipitation of iron from the medium is just visible at about three parts per million, and here also the inhibitory effect of excess iron is just detectable by decreases in growth. If citrate is present in the medium there is no precipitation of the iron, and the organism will tolerate the iron citrate complexes which ionize very slightly, until the iron content of the medium is quite high. Concentrations of iron in excess of 100 parts per million showed no inhibition of growth when citrate was present in the medium.

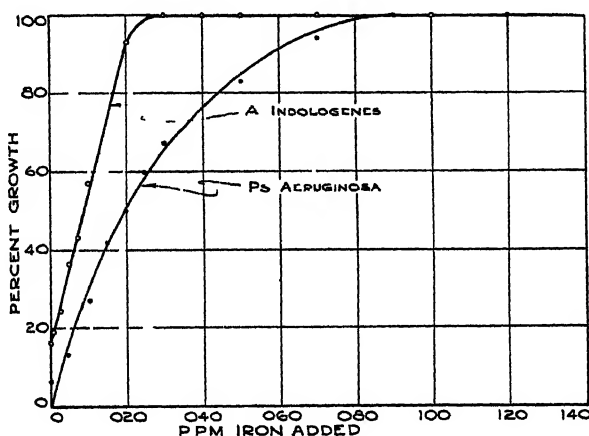


FIG. 1

It appears that the formation of ferric hydrate precipitate initiates bacterial growth inhibition by iron as borne out by a large number of experiments. The mechanism involved appears to be complex when viewed from a colloidal point of view. The colloidal sol stage through which the ferric hydrate passes produces minute particles of the solid hydrate which are probably adsorbed on the outer surface of the cell membrane, thereby interfering with cell wall permeability, the nutrition of the cell, excretion, and possibly respiration. An excess of solid ferric hydrate at the membrane surface will likely create an interference of ionic exchange as well as a possible interference with intake of substrate molecules and removal of waste products of fermentation and respiration. Excess solid iron in close proximity to the cell may cause an increase in solution of the iron brought about by the acidic products

of fermentation. The excess of dissolved iron might then enter the cell and bring about an ionic imbalance within the protoplasm itself.

Analysis of iron-deficient *Aerobacter indologenes* cells, which are white as compared with the normal cream colored cells, shows them to be very low in iron, but they always contain a measurable amount of the metal. It appears that a certain minimum quantity is necessary for the cell to reproduce. A deficiency of iron in the medium brings about not only low iron content (0.003% of dry weight), but also very low yields of cells (in the case of *A. indologenes* as low as 3% of optimum). The normal cells when grown on a medium containing 0.1 p.p.m. of Fe contain on an average 0.03% iron. When the cells are grown on a medium containing an excess of iron (5.0 p.p.m.), they contain a noticeable excess of iron (0.10%), which is evidently either adsorbed to the outer surface of the cells or is in some way stored within the cell protoplasm. The iron analysis in this work was done by a modification of the thio-glycolate method of Burmester (3).

Aerobacter indologenes has never been prevented from growing because of lack of iron, but after ten consecutive transfers on the deficient medium, growth was reduced from 7% of a normal control growth to 3%. Further refinement of the purification method, such as a continuous extraction on a specially designed extractor, followed by direct inoculation of low iron cells into the pyrex extraction thimble, thereby not requiring even one transfer of the medium to another vessel might result in a complete failure of appearance of growth.

Preliminary experiments on the Warburg respirometer have shown that the iron-deficient *A. indologenes* cells are almost catalase-free (about 3% of the normal activity). The cytochrome bands at 560 $m\mu$ and 590 $m\mu$ were not visible in the deficient cells, but were plainly observed in the normal ones. Other enzyme systems appear to be affected also. Work along these lines is now being carried out, as well as an extension of the growth work.

DISCUSSION OF EXTRACTION METHOD

Some mention of the theory of the extraction method is necessary in order to understand its limitations and advantages. 8-Hydroxy-quinoline is a well-known analytical reagent which forms inner complexes with a number of cations. Its properties are completely discussed by Prodinger (12). The reagent combines with the biologically important metals Cu, Fe, Mn and Zn throughout the pH range of the

average bacterial medium (pH 6-8). It was found that these metals, as well as others, form chloroform soluble, water insoluble complexes which are all extractable alike. For this reason it was thought best to add back the Cu, Zn and Mn, which are probably necessary in bacterial metabolism.

The chief limitation of the medium described is that it may be used only to study the simple, less fastidious organisms, such as those which have thus far been tested: *Aerobacter indologenes*, *Aerobacter aerogenes*, *Citrobacter intermedium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Phytomonas stewartii*, *Rhodococcus rhodochrous*, *Neisseria catarrhalis*, and *Klebsiella pneumoniae*. Yeasts and molds also grow well on the medium. It is believed that the medium can be extended to include those organic nutrients such as carbohydrates and amino acids that are not appreciably soluble in chloroform and might therefore be purified by the extraction method, and also such purified growth factors as biotin which are necessary in such small amounts that they may be added to the purified medium without appreciably increasing the iron content.

It appears that investigators have neglected the use of organic reagents for purposes of purification. There is a large number of these compounds and probably many of them have valuable properties. Oxine should have wide use in the removal of trace metals from many simple salts and organic chemicals used in biological work. If the substance to be purified has the following properties it might be purified by an oxine extraction.

- (1) Soluble in water, insoluble in chloroform.
- (2) Does not react with oxine or chloroform.
- (3) Gives a solution whose pH is or may be adjusted to the range of 4 to 10.
- (4) Does not form inner complexes or undissociable salts with the contaminating cations.
- (5) Does not exert a protective colloidal action on the contaminants, as in the case of proteins and lipides.

SUMMARY

An iron deficient medium is described which will produce cells of certain species of bacteria very deficient in iron. The medium contains between 0.0007 and 0.003 parts of iron per million parts of solution as calculated from growth curves. It is prepared by extracting the iron

complexes of 8-hydroxyquinoline with chloroform. Preliminary studies show that *Aerobacter indologenes* requires a minimum of 0.025 p.p.m. of iron in its culture medium for optimal growth, and that the iron-deficient cells produced in absence of iron, are very deficient in catalase and the cytochrome system. Oxine extractions are recommended for removal of traces of iron from a wide variety of compounds.

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$d(-)$ 3-Phosphoglyceric Acid

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Among the phosphorylated products originating from carbohydrates, *phosphoglyceric acid* has special significance: In its structure appears for the first time the carboxyl group which is a part of the final products of metabolism, *i.e.*, lactic acid, carbon dioxide, formic acid, and acetic acid. An extraordinary chemical resistance is confronted by a great biological changeability. Although the acid is easily converted biochemically, it is hardly affected by chemicals; this ester is unusually resistant (1) to acids and alkalis, and even to strictly chemical dehydration in pyrogenic reaction (2). The main properties of phosphoglyceric acid have been determined by Neuberg, Weinmann, and Vogt using synthetic *d,l*-3-phosphoglyceric acid.

When Nilsson (3) found phosphoglyceric acid among the products of metabolism of yeast cells in 1930 and considered it to be identical with the synthetic acid, whereas it was actually the *d(-)*3-phosphoglyceric acid, the significance of this compound in the carbohydrate metabolism of a great many kinds of cells has been recognized. Phosphoglyceraldehyde has to be considered as the immediate precursor of this acid. Neuberg and Kobel (4) succeeded 1933 in converting *d,l*-phosphoglyceraldehyde to *d(-)*3-phosphoglyceric acid, and Meyerhof (1938) isolated the phosphoric ester of the *d*-glyceraldehyde from the products of sugar degradation.

Besides for yeast (4), this important product has been established according to Embden and Meyerhof for the glycolysis of animal cells. Since Neuberg and co-workers had shown that not only yeast, but also certain bacteria (4) and green plants (5) have a phosphoglyceric acid metabolism, this behaviour has been often studied, in an especially careful manner by Werkman and co-workers (6). The importance of this substance is evident. F. and T. Schlenk (7) found an elegant application of the *d(-)*3-phosphoglyceric acid as substrate for the identification of adenosine-5-phosphoric acid and its homologues, based on the coenzyme properties of these compounds in the enzymatic splitting of the substrate; this is transformed by the enzymes present in the enzyme preparations by way of 2-phosphoglyceric acid and phosphopyruvic acid to pyruvic acid. In every respect the

3-phosphoglyceric acid has a special relation to the phenomenon of transphosphorylation.

This acid is not only interesting from the point of view of chemistry but also from that of physics. It has the ability of forming peculiar gelatinous salts which are very stable and characterized by surprising and extraordinary effects of double refraction (8).

Thus the preparation of this substance is not unimportant. Its ability to form a characteristic acid barium salt (1) which has been studied on the synthetic product, made possible a simple isolation (4) of the biological substance in the form of the splendidly crystallized salt: $C_3H_5O_7PBa + 2H_2O$, thus rendering unnecessary the complicated methods of isolation applied in the beginning by Nilsson for yeast and applied to animal material by the investigators of muscle chemistry (at first precipitation with coppersulfate-lime mixture, then transformation of the copper compound to the lead compound and subsequently to the untypical amorphous neutral barium salt, its transformation to the strychnine salt, etc.).

An additional essential fact was established by Neuberg with Kobel and Vercellone (4). It is not necessary to start from the comparatively costly hexosediphosphate, but one may proceed from a sugar phosphate mixture which by action of bottom yeast, capable of extra cellular accumulation of phosphorylated sugar, forms a solution containing *d*-fructose-1,6-diphosphate. After addition of acetaldehyde and of fluoride the Harden-Young ester in this solution is readily transformed to phosphoglyceric acid. The acid has been prepared frequently according to this inexpensive and convenient method; the only difficulty is that washed and pressed bottom yeast which has been used for this purpose is not available everywhere.

This difficulty can be overcome in a simple way by utilizing commercial American baker's yeasts, which are capable of phosphorylation. The following yeasts have this ability in fresh condition: Anheuser-Busch, Atlantic, Blue Ribbon, Federal and National Grain. The most consistent results have been achieved with National Grain yeast. The time required for complete phosphorylation was about 5 hours at 37°C. or 20–28 hours at room temperature. In the form of dried or acetone dried yeast there is hardly any difference between the various kinds of yeast, all of them phosphorylate completely within 45 to 90 minutes in the incubator; even Fleischmann's yeast which otherwise acts more slowly.

The declaration of Ostern and Guthke (9) (referring to bottom yeast), that for the preparation of phosphoglyceric acid following the method of Neuberg and

Kobel, it is advisable to start the fermentation with sugar solution alone, and to add the other ingredients later on, does not agree with our experience with top yeasts. It is not quite clear why a fermentation without addition of phosphate should improve the phosphorylating power of yeast. The application of sugar phosphate mixtures for this purpose, *i.e.*, the partial fermentation without addition of toluene was experienced early. The remark of the Polish authors may signify the revival of the fermentative power of yeasts which became weak and thus has no bearing on the formation of phosphoglyceric acid itself.

The following abstract from our records verifies these assertions:

Experimental

Experiment A: 600 cc. sugar phosphate solution (20 g. saccharose, 3.7 g. $\text{NaH}_2\text{PO}_4 + \text{H}_2\text{O}$, 1.1 g. NaHCO_3 per 100 cc. H_2O), 300 g. fresh National Grain yeast, 90 cc. carbon tetrachloride¹ are kept at room temperature for 25 hours, *i.e.*, until phosphorylation is completed (magnesia mixture test in the clearly filtered solution is negative). Subsequently are added: 840 cc. of 2% acetaldehyde solution, 140 cc. 0.2 *M* NaF solution, 1200 cc. 10% glucose solution, 380 g. fresh yeast, and 20 cc. CCl_4 . After thorough mixing and shaking the mixture again is kept for 24 hours at room temperature.

Because of the resistance of phosphoglyceric acid, the deproteinization may be carried out in the following simple way: After addition of 5 cc. glacial acetic acid the mixture is heated in a boiling water bath for half an hour, then filtered off or better centrifuged; to each 100 cc. of the clear filtrate 5 cc. of glacial acetic acid and 7 cc. of a 50% barium acetate solution are given, quickly filtered from a slight precipitation and the clear filtrate is kept in the refrigerator for 48 hours. Crystallization begins soon where scratched and is completed after this time. The substance is filtered under suction and washed with water. The crude yield obtained from 3700 cc. reaction mixture amounted to: 16.8 g. or 2.8 g. for 100 cc. of the phosphorylation mixture used.

The purification is carried out as follows: 1 g. of the substance is dissolved in 70 cc. 0.05 *N* HCl, warmed and filtered, then 140 cc. of alcohol are added to the clear filtrate. In a characteristic way the precipitate,

¹ As we have observed, benzene, petrol ether, xylene, trichloroethylene, brombenzene, carbon disulfide, amylalcohol, benzylalcohol can be used instead of the generally applied toluene. Ethyl ether and carbon tetrachloride are very effective plasmolytic agents and are equivalent or superior to the others. Chloroform fails, probably because of its protein precipitating effect, discovered by Salkowski (10).

which at first is milky, changes into silky lustrous crystals as soon as it is stirred. After standing for some time in the refrigerator the substance is filtered by suction and washed with dilute alcohol until free from chlorine. 4 g. of the crude product yields 3.5 g. of the pure acid barium salt of phosphoglyceric acid. The crude yield amounts to about 30% of the theory, if it is assumed (this assumption is, however, not correct), that the whole inorganic phosphate used is converted to hexose-diphosphate only, and that the added acetaldehyde allows the oxidative formation of 2 molecules phosphoglyceric acid, whereas without addition of acetaldehyde the oxido-reductive conversion to the 3-carbon-compounds makes possible the formation of one molecule of phosphoglyceric acid only. This yield is calculated without inclusion of the material contained in the mother liquor which may be considerable. But generally it is not worth while to use the large quantities of ethanol required for working up the mother liquor. This may be done as follows: To the filtrate from the precipitation with acetic acid and barium acetate, an equal quantity of alcohol (about 3300 cc.) is added and the mixture placed in the icebox for 24 hours. The precipitate formed is filtered off, washed with 50% alcohol and added to 440 cc. of water. In this operation a large part is dissolved. Now 2 *N* HCl is added until the solution is 0.05 *N*. The insignificant amount of undissolved product is removed by filtration over Fuller's earth and the acid barium salt is precipitated in the filtrate with 900 cc. of alcohol. After stirring for some time the characteristic crystals of the salt appear. These are filtered, thoroughly washed and dried. The additional yield amounts to 5.6 g. practically pure substance.

It may be noticed that even if the phosphorylation, *i.e.*, the starting formation of fructose-diphosphate has not been fully complete, the preparation of phosphoglyceric acid may be continued. If a small quantity of unesterified phosphate is present, it is removed in the purification process of the acid barium salt by precipitation in acid medium, and it is even unnecessary to eliminate small quantities of phosphoric acid with magnesium acetate as recommended by us previously. Small quantities of inorganic phosphate are esterified gradually during the fluoride period in the presence of glucose, but with another top yeast—Anheuser-Busch—some dephosphorylation seems to occur.

Experiment B: Identical with experiment A, except that the phosphorylation has been carried out in the incubator at 37°C.; this experiment gave lower yields, most probably on account of the fact, that at this

temperature a part of the primary phosphorylation products consists of mono-esters.

An ionic influence (11)- by applying KF or NH_4F instead of NaF—could not be established. We attempted also to achieve better results by increasing the concentration of acetaldehyde and of fluoride. This was suggested because the acid barium salt is not completely insoluble. Application of the same quantities of acetaldehyde and fluoride in double concentration, however, did not affect the yield. The addition of free glucose together with the fluoride may be omitted without influencing the result essentially.

Experiment C (serial): The results of repeatedly varied experiments are given here. The yields are calculated per 100 cc. of the sugar phosphate solution originally mentioned (3.7 g. $\text{NaH}_2\text{PO}_4 + \text{H}_2\text{O}$ or its equivalent in K- or NH_4 -salt). The additional yields from the mother liquor are not included. It is desirable to utilize the mother liquor for the preparation of the acid barium salt only in cases, when the first precipitate of the salt does not amount to more than 2 g. per 100 cc. sugar phosphate mixture.

1. 100 cc. sugar phosphate solution, 50 g. fresh National Grain yeast, 15 cc. CCl_4 are phosphorylated at room temperature, then 70 g. fresh yeast, 140 cc. 2% acetaldehyde solution, 23 cc. 0.2 M NaF solution and 3 cc. CCl_4 are added—*no addition of glucose*. The fluoride incubation also has been carried out at room temperature during 24 hours. Yield: 2.1 g. acid barium salt.
2. 100 cc. sugar phosphate solution² (with K_2HPO_4 , 4.65:100), 50 g. fresh National Grain yeast, 15 cc. CCl_4 , phosphorylated at room temperature, then 70 g. fresh yeast, 140 cc. 2% acetaldehyde, 23 cc. 0.2 M KF, 3 cc. CCl_4 ; room temperature. Yield: 2.8 g.
3. Identical with 2., but after completed phosphorylation addition of 200 cc. 10% glucose solution. Yield: 2.3 g.
4. 100 cc. sugar phosphate solution (K_2HPO_4), 50 g. fresh National Grain yeast, 15 cc. CCl_4 , room temperature; after 24 hours 70 g. yeast, 70 cc. 4% acetaldehyde, 12 cc. 0.4 M KF, 3 cc. CCl_4 ; room temperature. Yield: 2.6 g.
5. Identical with 4., but after completed phosphorylation, addition of 200 cc. 10% glucose solution. Yield: 2.4 g.
6. Identical with 3.; but after completed phosphorylation, addition of 100 cc. 10% glucose solution. Yield: 2.3 g.

² If K_2HPO_4 or $(\text{NH}_4)_2\text{HPO}_4$ is used, no bicarbonate is added.

(Various experiments which have in common the *phosphorylation at 37°C.* generally did not give constant results.)

Experiment D (serial): A series of experiments carried out with *dried yeasts*.

1. 100 cc. sugar phosphate solution, 50 cc. of water, 30 g. dried National Grain yeast, 20 cc. CCl_4^3 are completely phosphorylated at 37°C. within 1.5 hours: then addition of 50 cc. 2% acetaldehyde, 10 cc. 0.2 *M* NaF, 40 cc. of water; 3.5 hours at 37°. Further process as in A. Yield: 2.8 g. acid barium salt.
2. Identical with 1., but addition of 20 g. *glucose* in 40 cc. of water after completed phosphorylation. Yield: 2.7 g.
3. 5 g. *glucose*, 100 cc. sugar phosphate solution, 50 cc. of water, 40 g. dried National Grain yeast are kept fermenting for 20 minutes, then addition of 20 cc. CCl_4 and phosphorylation at 37° (1 hour 25 min.); 10 cc. 0.2 *M* NaF, 50 cc. 2% acetaldehyde, 40 cc. of water, 3.5 hours at 37°. Yield: 2.1 g.
4. Identical with 3., but addition of a solution of 20 g. *glucose* in 40 cc. of water after completed phosphorylation. Yield: 2.2 g.
5. Identical with 1., but sugar phosphate solution prepared with ammonium salt: 3.7 g. $(\text{NH}_4)_2\text{HPO}_4$: 100. Yield: 1.9 g.
6. Identical with 1., but sugar phosphate solution prepared with *K salt*: 4.65 g. K_2HPO_4 : 100. Yield: 2.7 g.
7. 100 cc. sugar phosphate solution (*ammonium salt*), 50 cc. of water, 40 g. dried yeast, 20 cc. CCl_4 , phosphorylated at 37° (1 hour 10 min.); then 25 cc. 4% *acetaldehyde*, 5 cc. 0.4 *M* *KF*, 3.5 hours at 37°. Yield: 2.0 g.
8. Identical with 7., but sugar phosphate solution prepared with *K salt* (K_2HPO_4). Yield: 3.2 g.
9. Identical with 1., but Fleischmann's yeast has been used; phosphorylation time 1 hour and 10 min. Yield: 2.8 g.

As shown, the yields obtained with dried top yeasts are in part better than those with fresh yeast. Though the whole procedure progresses faster, it will hardly be worth while to dry the yeast for this purpose and the use of suitable fresh top yeast will prove desirable.

It has been proved (4) that the acetaldehyde, which is used in the preparation of phosphoglyceric acid, may be substituted by other re-

³ If dried yeast is used the addition of a plasmolytic agent is not absolutely necessary; the same is the case when acetone dried baker's yeast or its active zymase extract is used for the preparation of phosphoglyceric acid.

ducible substances, for instance isovaleraldehyde or methylene blue. We found the use of *furfural* most convenient and manageable for this purpose; we applied 50 cc. of a 5% *furfural* solution for every 100 cc. of sugar phosphate mixture, at otherwise unaltered conditions. The yields were at least equal to those achieved with acetaldehyde. (At the end of the incubation time some of the *furfural* is unchanged, but a part of it certainly is reduced, which is demonstrated by means of the well known reaction of *furfuryl* alcohol with hydrochloric acid.)

Since we know only a few bio-analytical differences between top and bottom yeast, it should be kept in mind that a statement of Rapoport (12)—at least under the conditions mentioned—does not prove to be correct. He says: "Bäckereihefe unterscheidet sich von der Brauereihefe dadurch, dass sie Phosphoglycerinsäure auch bei Toluolvergiftung nicht in grösserem Masse ansammelt."

Analysis: The desiccator dried substance (in vacuo at 50°) has the composition: $C_5H_5O_7P\text{Ba} + 2 H_2O$ (Mol. wt. 357.5), as established by Neuberg and Kobel (4). The opinion of others that the salt crystallized with 1 or 1.5 or 3 moles of H_2O have been dropped; Kiessling and Schuster (13) finally found the same composition, and their statement concerning the rotation $[\alpha]_D = -13.6^\circ$ is in agreement with the observations of Neuberg and Kobel, who found $[\alpha]_D = -13.85^\circ$. The otherwise stated higher rotation presumably refers to a salt with less H_2O content.

0.1833 g. subs.: 0.1160 g. $Ba_2P_2O_7$; 0.1191 g. $BaSO_4$; 0.0590 g. $Mg_2P_2O_7$.

Calculated: $Ba_2P_2O_7$, 62.8; Ba 38.4; P 8.7%.

Found: $Ba_2P_2O_7$, 63.2; Ba 38.2; P 8.95%.

Our thanks are due to the manufacturers who provided us generously with the respective yeasts and to the Quaker Oats Company who provided us obligingly with furan derivatives.

SUMMARY

d(-)3-Phosphoglyceric acid can readily be obtained by means of American baker's yeasts under various conditions in the form of its acid barium salt without any special biochemical equipment.

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The Unautolyzable Protein of *Aspergillus sydowi*¹

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In previous papers (Woolley and Peterson, 1937) (14, 15) the isolation of thirteen amino acids (serine, valine, leucine, isoleucine, threonine, tyrosine, proline, tryptophan, aspartic acid, glutamic acid, arginine, histidine, and lysine) from the autolyzate of the mycelium of *Aspergillus sydowi* has been reported. During autolysis 63% of the nitrogen of the mycelium becomes water soluble. This figure is very close to the coefficient of digestibility of the proteins of *A. sydowi* and *A. fischeri* as determined by rat feeding experiments (Skinner, *et al.*, 1933) (12).

In the autolysis residue there remains some nitrogen soluble in 1% NaOH. In this paper is reported an attempt to purify the extracted protein, to test the action of proteolytic enzymes on the protein and to determine the amino acid content of the same.

EXPERIMENTAL

Preparation. The mould was grown and autolyzed as described in a previous paper (Woolley and Peterson, 1937) (14). After 5 days the autolyzate was filtered and the residue was well washed with water. The residue was then extracted with 1% sodium hydroxide solution (4 liters per kg. of mould) for 24 hours at room temperature. Three or four such extractions were usually made. Each extract was filtered and the protein was precipitated by addition of glacial acetic acid to a pH of 4.8-5. The protein would not redissolve at a lower pH as would that which was extracted by similar means from unautolyzed mould. The precipitate was washed with water, then alcohol, after which it was redissolved in 1% sodium hydroxide solution. The solution was filtered

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and the protein was reprecipitated by acidification, washed with water, alcohol and benzene. During the washing with benzene, the protein turned markedly darker, and when stirred in the benzene, the suspension took on the appearance of a dark brown solution. In the next step, washing with ether, the protein again assumed the original color, a light fawn. After one ether washing, the protein was dried *in vacuo*, pulverized, extracted continuously with ether, then acetone and finally chloroform, after which it was dried *in vacuo*. The yields were about 8 g. of protein per kilogram of dry mycelium.

Hydrolysis of the Protein by Various Enzymes. Lengthening the autolysis period to 2 months did not remove the resistant protein from the residue. As further evidence that the enzymes of *A. sydowi* did not hydrolyze this protein, an enzyme preparation was made, and it failed to produce any significant hydrolysis of the isolated protein. To prepare the enzyme solution, a fresh, wet pad of *A. sydowi* was frozen, thawed and then the liquor pressed out. This liquid was then dialyzed, filtered and concentrated until 1 ml. of solution corresponded to 1 g. of dry mould. Twenty ml. of enzyme solution was added to 200 mg. of wet protein, the pH adjusted to 5.5 and the mixture incubated at room temperature. As a check on the activity of the enzyme preparation a casein control was run at the same time. The 200 mg. of casein were solubilized within 2 days, but only 6% of the unautolyzable protein was dissolved in 21 days.

The action of other proteolytic enzymes on this protein was also tested. An enzyme solution was prepared from *A. parasiticus*, this mould being chosen because of its marked proteolytic properties (Berger, *et al.*, 1937) (1). In addition commercial preparations of pepsin, papain, ficin², pancreatin and trypsin were used. The enzyme concentrations used were such that they effected complete hydrolysis of 2% solutions of gelatin or egg albumin within 24 hours. Incubation was at 37°C., and pH values were as follows: 2 for pepsin, 5 for papain (with 0.001 *M* Na₂S), 5.5 and 7 for ficin (at pH 7 KCN was added to make the solution 0.001 *M*), 7 for *A. parasiticus* enzyme and 8 for trypsin and pancreatin. The results of the action of these enzymes on a 4% suspension of the unautolyzed protein are given in Table I.

Polysaccharide Content of the Protein. The possibility that the low nitrogen content of the protein was due to the presence of uncombined

² We wish to thank Dr. A. Walti of the Merck Research Laboratories, Rahway, New Jersey, for the gift of ficin.

carbohydrate was eliminated by performing a chloroform-gel separation (Sevag, 1938) (11) on a solution of the protein. This procedure did not raise the percentage of nitrogen in the product.

1. *Hexosamine*. A sample of the protein containing 0.2–1.25 mg. hexosamine was hydrolyzed by heating in a sealed tube with 4 *N* HCl at 115°C. for 8 hours and then treated as described by Hewitt [1938] (5) for the colorimetric determination of glucosamine. The color was allowed to develop for one hour and then read in an Evelyn photometer with color filter for 520 $m\mu$ wave length. A blank solution containing 1 ml. each of alcohol and concentrated HCl was set to read 100 in the

TABLE I
Action of Proteolytic Enzymes on the Protein

Enzyme*	Mould protein NH ₂ -N†	Time of enzyme action	NH ₂ -N liberated by enzyme	Hydrolysis
	mg. per ml	hours	mg per ml	per cent
Pepsin	2.8	65	0.07	2.5
Papain	2.8	68	0.06	2.1
Parasiticus	2.8	68	0.02	0.7
Pancreatin	2.8	70	0.20	7.0
Trypsin	2.8	70	0.27	9.6
Ficin (pH 5.5)	1.95	197	0.09	4.5
Ficin (pH 7.0 and 0.001 <i>M</i> KCN)	1.95	197	0.12	6.0

* Commercial preparations of enzymes were used except that from *Aspergillus parasiticus* which was prepared as described in the text. The enzyme concentrations were such that they effected maximum hydrolysis of 2% solution of gelatin or egg albumin within 24 hours.

† Amino nitrogen as determined by hydrolysis with 7 *N* H₂SO₄ for 7 hours at 120° C.

photometer. The readings obtained on the protein sample were compared with those of a standard glucosamine solution treated in a similar manner. The hexosamine content of the protein preparations is given in Table II.

2. *Reducing Sugar*. Treatment of the protein with 0.5 *N* H₂SO₄ for 6 hours at 100°C. liberated reducing sugar, which, calculated as glucose and determined by the method of Stiles, *et al.* [1926] (13) accounted for from 5.1 to 12.7% of the various preparations. Four of the six preparations yielded close to 10% or more reducing sugar. Longer hydrolysis did not produce any more sugar. These conditions liberated a small

fraction of hexosamine. The product after this treatment contained 11.8% nitrogen.

Phosphorus, Sulfur, and Purine Content of the Protein. Preparation B₁ contained a trace of sulfur, 1% phosphorus (Bodansky, 1912) (3) and 0.45% purine nitrogen (Graff and Maculla, 1935) (4). Other preparations contained varying amounts of phosphorus ranging from 0.29% for C₁ to 0.66% for B₂. Variations in the phosphorus content, yield of hexosamine and reducing sugar are understandable if the preparations consisted of several proteins rather than a single homogenous substance. Because of the method of preparation required, it is possible that such was the case.

TABLE II
Composition of the Resistant Protein of the Mycelium of Aspergillus sydowi

Run	Weight of mycelium	Extractions	Yield	N (ash-free basis)	Ash	Hexose-amine	Reducing sugar*
	<i>g</i>		<i>g</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	533	2 (A ₁)	8.4	11.2	3.9	†	9.35
2	1,139	1 (B ₁)	5.35	11.7	1.3	4.3	12.7
		2 (B ₂)	2.85	11.1	2.0	3.6	10.1
		3 (B ₃)	1.00	11.0	2.6	5.1	11.1
	
3	812	1 (C ₁)	5.13	11.3	0.5	1.8	5.1
		3 (C ₂)	1.69	11.2	1.2	2.6	6.1

* Liberated by 0.5 N H₂SO₄ at 100°C. in 6 hours, and calculated as glucose. After 26 hours hydrolysis preparation A₁ gave 9.27% reducing sugar.

† Not determined.

Amino Acid Composition. In order to study the amino acid composition of the protein, a 10 g. sample was hydrolyzed by heating it in 50 ml. of 7N sulfuric acid for 7 hours at 120°C. A large amount (3 g.) of humin was formed by this treatment. Seventy per cent of the nitrogen in the hydrolyzate was determinable in the Van Slyke apparatus. The sulfuric acid was removed with barium hydroxide, and the filtrate from the barium sulfate was fractionated by the same procedures as have been used in previous work for the separation of amino acids from hydrolyzates of the autolyzable proteins of mold mycelium (Woolley and Peterson, 1937) (14, 15). A brief statement will serve to indicate the sequence in which these procedures were applied. The dicarboxylic acids were precipitated with barium hydroxide and alcohol, and aspartic

acid was separated as the water-insoluble copper salt. The basic amino acids were then precipitated with phosphotungstic acid and separated by the well known silver salt procedure. The acids were finally obtained as arginine *flavianate*, histidine *diflavianate*, and lysine *picrate*. The solution containing the monoamino acids was concentrated to a small volume in order to crystallize most of the tyrosine. The filtrate was then extracted with butanol, and the copper salts of the extract were prepared and separated with water and with methanol. The proline fraction was treated with picric acid in order to isolate proline as the picrate. Leucine was obtained in pure form from the water-insoluble copper salts. The results of the fractionation are sum-

TABLE III
Amino Acids Isolated from the Hydrolyzate

Amino acid (or derivative)	Amount isolated	Criterion of purity
	mg.	
Copper aspartate	390	7.1% N
Arginine flavianate	756	M.P. 255-260°
Histidine diflavianate	126	M.P. 250°
Lysine picrate	540	M.P. 250°
Tyrosine	149	7.75% N
Leucine copper salt	1,050	8.76% N
Proline picrate	120	M.P. 154-155°

marized in Table III. The Hopkins-Cole test for tryptophan, when applied to a sample of the protein, was positive.

DISCUSSION

A unique property of our protein preparations is their resistance to enzymatic hydrolysis. In this respect they resemble untreated scleroproteins and many glycoproteins but differ from them in several respects chiefly in regard to their low sulfur content (cf. Block and Bolling, 1939) (2).

They are also distinctive in that they contain both firmly bound phosphorus and polysaccharide. The phosphorus is probably not esterified with an hydroxy amino acid as in the phosphoproteins since it is not set free by dilute alkali (Rimington and Kay, 1926) (10). The polysaccharide appears to be firmly bound as in such proteins as egg albumin,

seroglycoid, and ovomucoid. Release of the polysaccharide by digestion of the protein moiety—(Rimington, 1929) (9) (Neuberger, 1938) (8), (Hewitt, 1939) (6)—is not possible because of the resistance of the protein portion to proteolytic enzymes. It is possible that more than one protein in the mould remains unautolyzed. Alkaline solutions are known to cause marked changes in proteins (Neglia, *et al.*, 1938) (7), and it is possible that the protein as isolated is not the same as existed in the mould.

SUMMARY

An unautolyzable protein has been isolated from the mould *Aspergillus sydowi*. The yield was about 0.8 g. of protein per kilogram of dry mycelium. This protein was resistant to hydrolysis by pepsin, papain, trypsin, pancreatin, ficin, and an enzyme preparation made from *Aspergillus parasiticus*. The protein was insoluble in water but was soluble in dilute alkali. Various preparations contained an average of: 11.3% N, 1.9% ash, 3.5% hexosamine, 9.1% reducing sugar, calculated as glucose, 0.7% P, 0.5% purine N, and a trace of sulfur. That the carbohydrates were combined with the protein was indicated by resistance to separation when subjected to a chloroform-gel fractionation.

Histidine, arginine, lysine, tyrosine, aspartic acid, leucine, proline and tryptophan have been isolated from hydrolyzates of the protein.

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The Gums from the Carob Bean, *Ceratonia Siliqua*, L.¹

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The carob bean, native to the Mediterranean region where it is used as a food under the name of St. John's bread, yields a seed gum which is finding increasing uses in the United States for such purposes as the sizing of textiles, the tanning of leather, in the dyeing and printing of fabrics, as well as in mucilages, facial creams, chewing tobacco and in pharmaceutical products. One and half million pounds have been imported annually into the United States.

The purpose of this investigation was to study certain of the properties and the chemical structure of this gum with the thought that some similar native seed gums might be substituted for it.

HISTORICAL

In 1897, Effront (1, 2) investigated the mucilaginous carbohydrate from the carob bean, which he called "caroubine"; by hydrolysis he obtained a sugar which he called "caroubinose" and which van Ekenstein (3) claimed to be mannose. Marliere (4), however, reported that the carbohydrate consisted of glucose, fructose, and galactose, and that mannose was absent. Bourquelot and Herissey (5) reported that the gum was composed of 83.5 per cent mannose and 16.5 per cent galactose. Iglesias (6) stated that the gum consists exclusively of four molecules of mannose to one of galactose and demonstrated that no uronic acids were present. Gutbier, *et al.* (7, 8) found the gum negatively charged in water and studied its protective action on selenium sols.

Hart (9) found that a 1 per cent solution of the gum was very viscous and that a 5 per cent solution set to a gel. A 0.75 per cent solution was 35.7 times more viscous than water. Williams (10) and Hart (9) ob-

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served that the gum was very sensitive to borates. When borax was added to a sol of this gum there resulted an irreversible, clear gel which did not wet glass and several other substances. Williams ascribed the formula $C_6H_{10}O_5$ to the gum.

EXPERIMENTAL

1. *Purification of the Gum*

The commercial product was stirred into water to which more solvent was added to make a 0.2 per cent solution. The solution was filtered hot from any foreign material. When cooled an equal volume of 95 per cent ethanol was added. This yielded a stringy precipitate which floated on the surface and a portion which settled out as a fine powder. Less alcohol yielded more of the material in the powdered form; more alcohol resulted in a thick clot. The floating material was removed, expressed to remove excess liquid, washed in turn with absolute alcohol and with ether, and dried in vacuo. The material which had settled out was dried in the same manner. The two products showed the same gelling properties and gave identical analytical results. The purified dried gum had a moisture content of 2.8 per cent as determined in a vacuum oven gradually raised from 80° to 98°C. over a period of five days and its ash content was 0.085 per cent. Upon electrodialysis the ash was reduced to 0.05 per cent and a sol was free of any acidity indicating the absence of uronic acid (McLaren, 11).

2. *Qualitative Tests*

The gum was hydrolyzed by dilute H_2SO_4 , the acid removed by $BaCO_3$ and the sugars identified by osazone tests (Morrow and Sandstrom, 23). Only galactosazone and glucosazone (mannosazone) were found. Mannose was shown to be present by the formation of mannose hydrazone in the cold. Glucose and fructose were known to be absent by the failure to form glucosazone after the removal of the mannose hydrazone. Arabinose was further shown to be absent by a negative diphenylhydrazine test, and xylose by a negative cadmium bromide-cadmium xylonate test (23).

3. *Quantitative Tests*

Fructose (and other ketoses) were further shown to be absent by quantitative determinations for total reducing sugars by the ferricyanide method (25) and for aldoses by the alkaline hypiodite method (19).

The values obtained were 3.216 g. and 3.166 g. for equal aliquots which are identical within experimental errors. No uronic acids were found, using the method of Lefevre and Tollens (23, p. 40). Pentoses also were found absent by the A.O.A.C. phloroglucinol method and the thiobarbituric acid method (Bailey, 24).

Mannose was determined by the mannose-hydrazone method, and galactose by the mucic acid method (Morrow and Sandstrom, 23). One gram of the gum was taken and hydrolyzed by dilute acid. A slight residue was filtered and the filtrate was neutralized with BaCO_3 in cases where H_2SO_4 was used and by Ag_2CO_3 or Na_2CO_3 where HCl was used. Aliquots of the neutralized hydrolyzate were taken for reducing sugar determinations by the ferricyanide method (25) and for galactose and mannose. Better results are obtained with HCl . Typical results are shown in Table I. The values for galactose are very likely low since this method usually gives low results. If we consider that mannose

TABLE I
Analyses of Acid Hydrolysate of Carob Bean Gum

Grams gum used	Hydrolysis agent	Time of hydrolysis hours	% of theoreti- cal sugars*	% mannose	% galactose	Ratio mannose to galactose
1.0267	0.2N HCl	4	91.1	74.7	17.5	4.3
1.1634	0.1N HCl	24	101.9	75.5	18.5	4.1
1.0877	0.1N HCl	24	95.2	76.5	16.5	4.5

* Determined and calculated as glucose.

comprises 75 per cent of the gum and that the rest is galactose, the ratio of mannose to galactose will be three to one. Specific rotation measurements give a ratio of about 3.5 (see later).

4. Cleavage by Periodic Acid (12, 13, 14, 15)

0.5569 g. of gum was taken and 25 cc. of 0.4827 *M* HIO_4 was added to it in a glass-stoppered Erlenmeyer. At certain intervals aliquots were taken out and the amount of periodic acid remaining was determined by the arsenite method of Fleury and Lange (13). The results are shown in Table II. Since 1 mole of periodic acid is consumed per hexose unit a 1,2 or a 1,4 linkage is indicated. About 90 per cent of the weight of the original gum was obtained as the cleaved aldehyde product.

5. Phenylhydrazine Derivatives from the Gum Aldehydes

1.0 g. of the cleaved product was hydrolyzed with 15 cc. of 0.5 *N* H_2SO_4 , and a slight dark residue filtered off. The filtrate was neutralized with BaCO_3 and the excess barium was removed by H_2SO_4 . To one-half of the filtrate 1.5 cc. of phenylhydrazine was added together with a little alcohol and the solution heated at 60° for two hours. A yellowish brown precipitate was formed. This was filtered and dried. To the residue cold absolute alcohol was added. A part of the residue dissolved; this was recrystallized. M.P. $125\text{--}127^\circ\text{C}$., decomposes with gas evolution at 132. The residue was dissolved in ethyl acetate and thrown out by water. M.P. with frothing at $129\text{--}132^\circ\text{C}$.

TABLE II

Rate of Consumption of HIO_4 by Carob Bean Gum

Time hours	Molecules of HIO_4 Consumed per hexose unit
0.5	0.60
3.0	0.88
6.0	0.92
21.0	0.997 or 1.00
29.5	1.08

6. Oxidation of Gum Aldehydes

4.7 g. of the cleaved gum was taken and hydrolyzed with 60 cc. of 0.06 *N* H_2SO_4 . The solution was neutralized with BaCO_3 and treated with Norite. Seven cc. of bromine was added to the filtrate which was allowed to stand in the dark for 19 days, after which the solution showed only a slight reducing power towards Fehling's solution. The excess bromine was removed with air and the solution neutralized with BaCO_3 . From the filtrate a brucine salt was prepared as a slightly yellow crystalline product which darkened at 230° and melted with decomposition at $259\text{--}261^\circ$, $[\alpha]_D^{25} = -16^\circ$ ($C = 0.4344$). An authentic sample of brucine tartronate darkened at 210° and melted at 244.5° , $[\alpha]_D^{25} = -17.3^\circ$ ($C = 0.5420$).

The barium precipitate was likewise converted to its brucine salt. M.P. $215\text{--}220^\circ$, $[\alpha]_D^{25} = -30.5^\circ$ ($C = 0.7160$). An authentic sample of brucine glycerate (from racemic glyceric acid) was prepared. M.P. = $219\text{--}220^\circ$, recorded in literature as 220° , $[\alpha]_D^{25} = -33^\circ$. Mixed M.P. = $215\text{--}216^\circ$.

7. *Molecular Size by the Alkaline Hypodite Method (19, 20)*

0.8 g. of gum was taken and dispersed in about 50 cc. of water; 20 cc. of 0.1 *N* NaOH and 25 cc. of 0.1 *N* iodine were added and the mixture was allowed to stand in a glass-stoppered Erlenmeyer. After 5 hours the solution was acidified and the iodine titrated with 0.1 *N* Na₂S₂O₃. The iodine number was calculated. This divided into 20,000 gave the molecular weight. The iodine numbers found were 3.89 and 3.25 corresponding to molecular weights of 5141 and 6154 which represent 32 and 38 hexose units, respectively; mean value, 35 units.

8. *Molecular Size by the HIO₄·HIO₃ Method ("Short-Time" Method, 12, 13, 14, 15)*

Approximately 0.7 g. of gum was weighed into a 125 cc. glass-stoppered Erlenmeyer flask. To this was added 10 cc. of 0.5 *M* HIO₄ (10 per cent excess), and the mixture allowed to stand for five or more days in the dark. This was then filtered through a plug of glass wool and asbestos, the cleaved gum was washed thoroughly of all reagent and the filtrates combined. The combined filtrate was heated on a water bath kept at a temperature of between 60 and 62°C. for six to seven hours, with a slow current of air blowing onto the surface. This was then cooled and diluted to 100 cc. This solution was then used to determine the HIO₄ and the HIO₃ contents. Periodic acid was determined by the method of Muller and Friedberger (26) with 25 cc. of the solution. Knowing the amount of periodic acid taken, the periodic acid consumed was determined. Also the iodic acid remaining was determined upon 10 cc. of the filtrate by adding 5 cc. of *N* H₂SO₄ and 5 cc. of 20 per cent KI solution and titrating the resulting iodine with 0.1 *N* Na₂S₂O₃.

$2.5(\text{HIO}_4 \text{ consumed} - \text{HIO}_3 \text{ found}) = \text{HCOOH in moles}$

$$\text{M.W.} = \frac{3}{\text{HCOOH}} \times \text{wt. of gum}$$

The results are shown in Table III and indicate an average molecular weight corresponding to 35 hexose units.

9. *The Sodium Periodate Methods*

(a) *With Methyl Orange as Indicator.* 0.9133 g. of gum was weighed into a 125 cc. glass-stoppered Erlenmeyer and 25 cc. of 0.5 *M* sodium periodate added to it and the mixture allowed to stand in the dark for 20 days. The resulting formic acid was then titrated with 0.05 *N*

NaOH, using methyl orange as indicator. A blank was treated similarly. The result was a molecular weight of 2693 or 17 hexose units.

(b) *With Phenolphthalein as Indicator.* Three samples of approximately 0.18 g. of gum were weighed into 125 cc. glass-stoppered Erlenmeyers and 5 cc. of 0.5 *M* sodium periodate added to each and the mixture allowed to stand for three days in the dark. Two blanks of 5 cc. were treated similarly. By this time 1.12 molecules of periodate had been consumed per hexose unit. To each flask seven drops of ethylene glycol were added to destroy the excess of sodium periodate. After 24 hours the solutions were titrated with phenolphthalein as indicator. The blanks require 0.10 cc. of alkali. The results are tabulated in Table IV.

(c) *By Iodometry.* 0.4441 g. of gum was weighed into a 125 cc. glass-stoppered Erlenmeyer and 15 cc. of 0.3 *M* sodium periodate added to it

TABLE III
Molecular Size Determinations by the HIO_4 - HIO_3 Method (Short Time)

Wt. of gum	HIO_4 , consumed/ hexose unit	Molecular Weight	Sugar units	
0.7030	1.03	6113	38	
0.7082	1.03	4862	30	
0.7015	1.04	6047	37	Av. = 35
0.7063	1.06	5845	36	
0.7063	1.06	5297	33	

TABLE IV
Molecular Size Determination by the Sodium Periodate Method Titrated Against Phenolphthalein

Wt. of gum	cc. of 0.0492 <i>N</i> NaOH	Molecular Weight	Sugar units
0.1833	4.53	2523	16
0.1853	4.53	2522	16
0.1823	4.52	2515	16
0.9133	25.20*	2693	17

* Without removal of periodate, titrated against methyl orange; blank = 4.52 cc.

and allowed to stand in the dark. A blank of 15 cc. of the reagent was also set aside. After five days 0.3 cc. of glycol was added to the blank and 0.15 cc. to the actual run. At the end of 24 hours the blank showed no presence of periodate. To each was then added 1 cc. of 20 per cent KI solution and the iodine liberated was titrated with 0.0137 *N* $\text{Na}_2\text{S}_2\text{O}_3$.

The blank required 0.90 cc. while the run required 46.90 cc. This gives a molecular weight of 2123 or thirteen hexose units.

10. The "Short-Time" HIO_4 - HIO_3 Method on Glucose

This is the method as used on the carob gum. A 0.2420 g. sample of *d*-glucose (Mallinckrodt analytical reagent) was weighed into a 125 cc. Erlenmeyer and 15 cc. of 0.4579 *M* HIO_4 added. This solution was treated in the same manner as for the gum; 5.1 molecules of periodic acid were consumed per glucose molecule and 2.6 molecules of formic acid were accounted for. This means that half of the formic acid was not oxidized in the time involved.

11. The "Long-Time" HIO_4 - HIO_3 Method on Glucose

0.2476 g. and 0.2473 g. samples of glucose were weighed into 125 cc. glass-stoppered Erlenmeyers and 15 cc. of 0.4987 *M* HIO_4 added to them and allowed to stand for 5 days at a room temperature of about 25°C. This was placed in an oven kept at 31–34°C. for 12 days, when the temperature was raised to 40°C. for 5 days, then raised to 60°C. in 4 days and kept there for 6 days. At 20 days the stoppers were opened and the iodine allowed to vaporize off. Upon again closing the flasks iodine re-appeared. This process was repeated three times, until finally after 30 days no more iodine was given off at 60°C. over a two-day period. The residual HIO_4 and HIO_3 present were determined. 5.45 molecules of periodic acid had been consumed per glucose molecule. The amount of formic found was 5.3 and 5.2 molecules per glucose molecule. These experiments show that the "short-time" HIO_4 - HIO_3 method as used on the gum very likely did not account for all the formic acid formed from it. Since, however, the method gives consistent results, the conditions used were probably such that a consistent fraction of the formic acid was oxidized. On this basis a corrected value for the "short-time" version on the gum is one-half of the value obtained, or about 18 hexose units. This corrected value will then check the values given by the sodium periodate methods.

12. Optical Rotation Studies

Varying concentrations of the gum and HCl acid were used and the rotations taken at frequent intervals. $[\alpha]_D^{30}$ for the initial gums were: +42.45°, +43.26°, and +45.44°. After heating for 6 or 7 hours the values reached +26.53°, +29.50°, and +28.13°. These values agree well with one calculated for mixtures of one galactose unit to 3 and to 4 mannose units (calculated values +31.07° and +27.28°, respectively).

It should be recorded that lower values for $[\alpha]$ were obtained after partial hydrolysis.

13. Partial Hydrolysis of the Gum

1.5 g. of gum with 35 cc. of 0.2 *N* HCl was heated in a boiling water bath for 75 minutes. An osazone test at this time showed only galactose with no mannose. The hydrolyzate was neutralized with Na_2CO_3 and 4 volumes of absolute alcohol were added, on which a white amorphous precipitate resulted. Yield = 0.5 g. The molecular size of this product was determined by the alkaline hypiodite method, and gave a value of 13 hexose units, which is the maximum size of the residual mannan portion of the gum, since probably not all the galactose has been hydrolyzed off. This run was repeated on 2 g. of gum with 35 cc. of 0.2 *N* HCl hydrolyzed in a boiling water bath. Osazones were prepared at intervals. After 105 minutes galactose but no mannose was being liberated, but after 110 minutes both sugars were appearing. At this point the hydrolyzate was neutralized and evaporated in vacuo to about 15 cc. To this was then added 150 cc. of alcohol, on which a white amorphous, hygroscopic precipitate resulted. Yield = 0.3 g. The molecular size of this product was determined by the alkaline hypiodite method and gave a value of 8 hexose units.

A third lot of the gum was hydrolyzed until mannose began to appear. The product was precipitated with alcohol, and purified by reprecipitation from water with alcohol, $[\alpha]_D^{27} = 4.0^\circ$. The material was completely hydrolyzed and the product again polarized, $[\alpha]_D^{27} = +13.8^\circ$, which agrees fairly well with the reported value for mannose, $[\alpha]_D^{27} = +14.6^\circ$. Thus the residue remaining when mannose begins to be liberated appeared to be essentially a mannan.

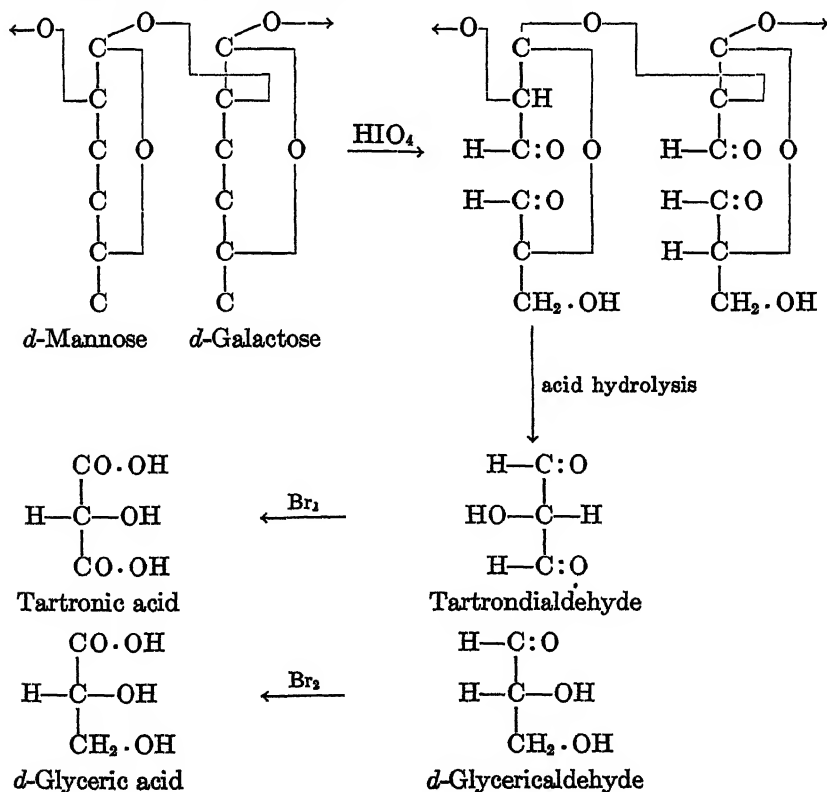
DISCUSSION

The carob gum differs from many gums in that it contains no pentoses, pentosans or uronic acids. It is composed of 1 molecule of galactose to approximately 3.5 molecules of mannose as evidenced by the determination of the former as mucic acid and by the latter as the hydrazone. The specific rotation of a completely hydrolyzed gum also gives a value supporting this.

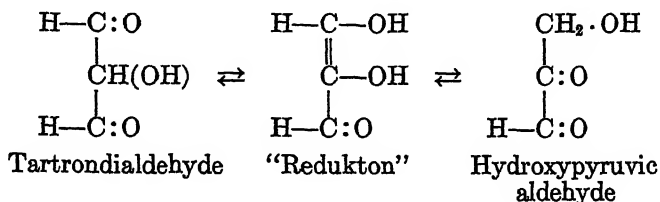
The gum has no reducing property and is readily hydrolyzed by acids; these facts indicate that the linkages between the hexose units are glycosidic. Such linkages, together with evidence to be cited later, lead to the assumption of long chains. If we may assume the generally occurring pyranoside structure for each hexose unit (which was not in-

vestigated here) there remain 4 hydroxyl groups which can serve to tie one unit to the next hexose unit at the glycosidic position, namely on carbons 6, 4, 3, and 2. The 1,6 linkage should consume 2 moles of periodic acid per hexose unit; the 1,4 and 1,2 linkages would each require 1 mole; and the 1,3 linkage would not react with periodic acid since there are no two neighboring hydroxyl groups (12, 13, 14, 15). Oxidation with periodic acid consumed 1 mole of oxidant per hexose unit, thus limiting the linkages to either the 1,4 or 1,2 linkage.

To decide between these alternatives the cleaved gum was examined for the aldehydes and the acids formed. The 1,4 linkage would involve splitting out carbon number 3 as formic acid and would yield 2 moles of glyoxal and 1 mole each of *d*-erythrose and of *d*-threose. Upon bromine oxidation these would yield 2 moles of oxalic acid and 1 mole each of *d*-erythronic and *d*-threonic acids. Such was not the case. The products obtained can be interpreted on a 1,2 linkage.



When the cleaved gum was hydrolyzed and the phenylhydrazones made from the aldehydes, two fractions of melting points 125–127° and 131–132° were found. The phenylhydrazones of glyoxal, *d*-erythrose and *d*-threose melt at 169–170° (also reported as 171–172° and 178–179°), 164° and 164°, respectively. Glycericaldehyde phenylhydrazone has a melting point of 131°. Tartronaldehyde is unknown. Euler and Martius (16) regarded “redukton” as the ene-diol of tartrondialdehyde. Furthermore, the ene-diol of hydroxymethyl glyoxal, or hydroxypyruvic aldehyde, would have the same formulas as “redukton” (17).

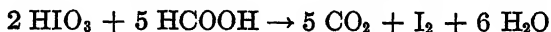


Evans and Waring (18) found the phenylhydrazone of hydroxypyruvic aldehyde to have a melting point of 131°, and found it to be identical with that of glyceric aldehyde and dihydroxy-acetone. Norrish and Griffiths (17) reported the melting point of the phenylhydrazone of hydroxypyruvic aldehyde to be 132°. Our melting points lie closer to those for glyceric aldehyde and for tartrondialdehyde or its related products.

When the gum aldehydes were oxidized with bromine, two brucine salts were obtained, one with a melting point and a specific rotation of 215–220° and –30.5°, and the other with 259–261° and –16°, respectively. The values for brucine *d*-glycerate are 220° and –33°, while those for brucine tartronate are 244–245° and –17.3°, respectively. A mixed melting point determination of our compound suspected of being brucine *d*-glycerate with an authentic sample of brucine glycerate (from *dl*-glyceric acid) gave a value of 215–216°. The products obtained conform to those expected from a 1,2 linkage between hexose units.

Four methods were used to determine the molecular size. Willstätter's (19, 20) alkali hypiodite method measures the 1 end functional aldehyde group and led to a value of 35 hexose units; however, the reliability of this method has been questioned (21). The periodic-iodic acid method depends upon the fact that each hexose unit in the interior of the gum consumes 1 mole of periodic acid, the free aldehyde end hexose requires 3 moles of periodic acid and produces 2 moles of

formic acid plus 1 mole of formaldehyde, while the hexose unit at the other end of the chain reacts with 2 moles of periodic acid and yields 1 mole of formic acid. Thus each mole of the gum yields 3 moles of formic acid, which in turn, react with the iodic acid produced thus:



Thus the difference between the iodic acid produced by the consumption of periodic acid and that found after the reaction is a measure of the formic acid produced and hence the molecular size. By a "short-time" procedure a value of 35 hexose units was obtained. However, this method when applied to glucose gave only 2.5 moles of formic acid per mole of glucose instead of the theoretical 5 moles. A "long-time" method, which permits a longer time for the oxidation, accounted for all 5 moles of the formic acid. This "long-time" method was not applied to the gum but the correction factor of 0.5 was applied to the results.

Sodium periodate was used in a similar manner but titrating the formic acid produced with methyl orange and with phenolphthalein following destruction of the excess reagent with ethylene glycol. An iodometric titration was also employed giving molecular weights corresponding to 17, 16, and 13 hexose units.

In comparing the results from the four methods for determining the molecular size of the gum we obtained a value of 35 hexose units by the hypiodite method which method is known (21) to give high values. By applying the correction factor established on glucose the periodic-iodic method gives a value of 17 or 18 which agrees reasonably well with those found by the alkaline periodate method. A determination of the osmotic pressure using the osmometer of Dobry (22) gave a molecular weight of approximately 1300 hexose units. It is to be emphasized that this latter is a measure of the number of particles comprising the sol and not of the chemical entities which go to make up these particles.

Enzymatic studies gave inconclusive results as to the type of glycosidic linkages involved (α or β). Takadiastase hydrolyzed the gum rapidly, while emulsin and saliva gave little or no hydrolysis. Optical rotation studies gave very interesting results. The gum has a specific rotation of $+43^\circ$ and when hydrolyzed gave an equilibrium value of about $+28^\circ$. This in itself would suggest α linkages. However, during the hydrolysis the optical rotation drops rapidly from $+43^\circ$ to nearly zero and then rises to the equilibrium value of $+28^\circ$. This would indicate that the linkages are not entirely in the α form. If we postulate α linkages for

the galactosides, and β linkages for the mannosides the drop and the rise may be accounted for. For in such a case, the initial splitting off of the α galactosides (see later) will bring about a lowering of the rotation, which is further lowered by the remaining β -mannosides. Later the hydrolysis of the β -mannosides will result in an increase of the rotation to the final equilibrium rotation. To supplement this point, the optical rotation of the partially hydrolyzed gum taken to be pure mannan was determined. It gave a specific rotation of $+4^\circ$ or less. This low value may or may not indicate β linkages for the mannan portion (β -*d*-mannose $[\alpha]_D = -17^\circ$) but it helps to explain the low drop obtained. When this was hydrolyzed it gave an equilibrium value of $+13.8^\circ$ which checks closely the equilibrium value for mannose of $+14.6^\circ$.

If we assume that the gum is made up as a linear polymer of 3 or 4 mannose units to one of galactose to a total length of approximately 18 units, there remains to be determined how these sugar units are distributed along the chain. The various possibilities were considered in turn, the best picture is represented by a chain of 6 or 8 mannose units tied to 2 galactose units, with repetition of this unit. This is supported by the fact that all the galactose is liberated by hydrolysis before any mannose appears. The resulting mannan gum was found to contain 6 mannose units at the point when mannose was beginning to be liberated. The indefinite value of 6 to 8 mannose units may be due to a variation between 6 to 8 mannoses or it may be due to inadequacies in the methods of analysis. Possibly it may not be as low as 6, since a little mannose had already begun to be liberated. How much had come off is difficult to say, for only 5 minutes had elapsed since the preceding test which had showed no mannose, in a total hydrolysis time of 110 minutes. It would probably be safe to say that at the moment before the first mannose was released the value would not be much higher than six, it probably may be seven. This would check with the analytical results of a ratio of mannose to galactose of 3.5. There is, of course, no reason for supposing that there cannot be some variation, and that instead of exactly 6, 7, or 8 mannose units separating every 2 galactose units, there may be a mixture of all three kinds, and perhaps once in a while, even 3 galactose units.

No attempt was made to determine whether the chain begins and ends with mannose or with galactose. However, the optical rotation data may afford an insight into this question. If the immediate drop in optical rotation is due to α -galactosides being hydrolyzed, it is probable that one end of the chain is occupied by galactose. Such a chain may

then hydrolyze to give the experimental results obtained. It may do so by first splitting off the galactoses at the end, and next from the inside where the galactosidic linkages are located, as galactans are known to hydrolyze more easily than mannans (*e.g.* galactans of larch as compared with mannans of ivory nuts). Further, if the galactosidic linkages are α and the mannosidic are β , by analogy to starch and cellulose, we may expect the galactosidic linkages to hydrolyze off more easily than the mannosidic linkages. This will result in a rapid increase in the reducing power, a lowered viscosity, and the release of free galactose before mannose.

SUMMARY

1. The carob bean gum consists of 3 to 4 molecules of mannose to 1 of galactose. Uronic acids and pentoses are absent.
2. Evidence was presented to indicate the presence of 1,2 linkages.
3. It was shown that the galactoside linkages are probably in the α form while the mannoside linkages are probably in the β form.
4. A modified $\text{HIO}_4\text{-HIO}_3$ method for determining the molecular size of this polysaccharide was developed. When corrected this gave a value of approximately 18 hexose units for the molecular size.
5. Three sodium periodate methods gave values of 17, 16, and 13 hexose units for the molecular size.
6. Evidence was given to show the relative positions of the various sugar units. It is probable that at least one of the two ends is occupied by galactose. Reasons were advanced to show that the molecule consists of a mannan chain of 6 to 8 mannose units followed by 2 galactose units, and so on to a total of about 18 hexose units.

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Adenochrome, a Glandular Pigment in the Branchial Hearts of the Octopus¹

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A recent publication from this laboratory (1) which dealt chiefly with the carotenoids in the digestive gland of the two-spotted octopus, *Paroctopus bimaculatus*, gave passing reference to a blood-red, water-soluble pigment encountered in the "kidneys" of this species. Actually, the structures in which the pigment occurs are the branchial hearts, a pair of small, purple, oval-shaped pulsating organs, situated outside of the large kidney-sac proper, one at the base of each gill. These branchial hearts are partly muscular, serving to pump blood into the gills, but composed largely of spongy, glandular tissue, supposedly of excretory function. In the glandular cells are stored considerable quantities of the pigment in the form of deep garnet-red cell-inclusions. The properties of this pigment and its deposition exclusively in tissue of supposed excretory role lead us to believe it to be a metabolic waste product (2-7).

PHYSICAL PROPERTIES AND CHEMICAL BEHAVIOR OF THE PIGMENT

A. Appearance, color and solubility: The name adenochrome which we have given to this pigment arose from the fact that its only locus of deposition seems to be in the glandular tissues of the branchial hearts. Here it shows a strikingly uniform distribution throughout the component cells (see Fig. 1), imparting a purplish color to the whole organs.

Fresh sections placed upon microscope slides yielded their pigment slowly to water, rapidly to dilute ammonia, leaving in each cell one or more colorless granular masses which formerly incorporated the colored material.

¹ Contributions from the Scripps Institution of Oceanography, New Series No. 184.

Adenochrome is fairly soluble in water, especially if warm, yielding wine-red colloidal solutions which manifest a Tyndall beam and from which the pigment fails to diffuse through commercial animal-membranes, even against a gradient of saturated NaCl solution. It is not precipitated from neutral aqueous solutions by boiling or by saturation with NaCl or $(\text{NH}_4)_2\text{SO}_4$. It is readily soluble in alkali at all concentrations.

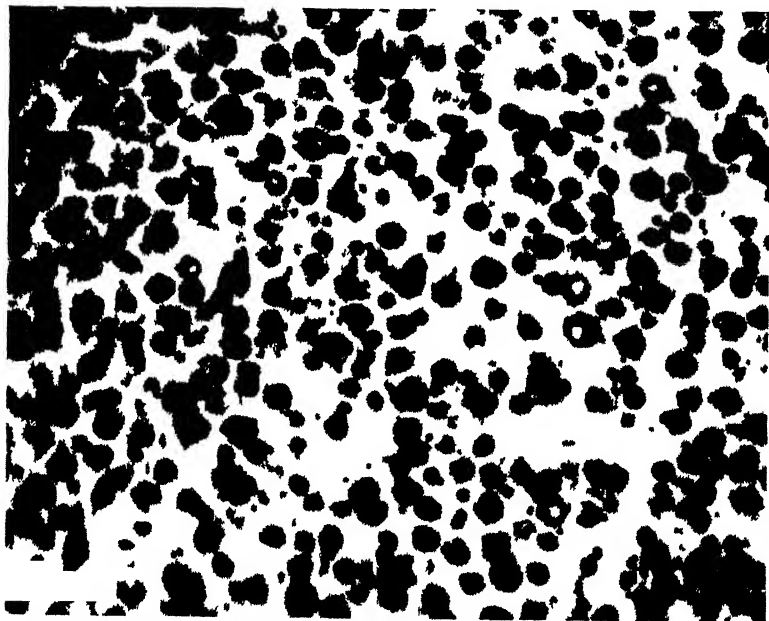


FIG. 1. Pigment inclusions in glandular cells of branchial heart tissue.
(\times ca. 270)

The addition of dilute acid or of small amounts of alcohol or acetone to a dilute solution of the pigment causes a change from wine-red color to a purplish, turbid appearance, and results in the eventual precipitation of the pigment in amorphous flocks.

Appreciable concentrations of alcohol (30% to 50% by volume) bring about the ready and complete precipitation of adenochrome, if a trace of acid is added. Neutral solutions require greater amounts of the precipitant, and alkaline solutions have failed to yield a precipitate by the addition of alcohol. These observations suggested the weakly

acidic nature of adenochrome, which was later confirmed by titration with dilute alkali.

B. Preparation and yields of pigment: On the basis of the solubility of adenochrome in water (ca. 0.7 g. per l.) and aqueous systems and its insolubility in alcohol, a simple procedure was achieved for its isolation in nearly quantitative amounts. From weighed specimens sacrificed by chilling in a refrigerator, the branchial hearts were carefully removed, weighed and extracted either wet or following drying *in vacuo* over CaCl_2 at room temperatures. (Oven-drying at 108°C . was found to result in partial decomposition, giving a somewhat charred residue.) The mass was then ground in dilute NH_4OH or Na_2CO_3 solution (approx. 0.5%) until fine comminution of the material had resulted. The aqueous extract was decanted and the remaining solid material re-extracted a few times until it assumed a pale color and yielded no more pigment to the dilute alkali. Rendering the combined extract faintly acidic by the addition of dilute acetic acid was useful in promoting the complete coagulation of all protein material by boiling at this stage. The system was then cooled, rendered faintly alkaline again with dilute NH_4OH or Na_2CO_3 , and filtered from the coagulum through paper. The filtrate was rendered barely acidic with dilute acetic acid, treated with an equal volume of 95% ethanol, and the resulting purple flocks of pigment were separated by centrifugation. Washing of the precipitated material with 50 to 75% ethanol, and sometimes with absolute ethanol and with ether, was followed by redissolution in dilute alkali, acidification with dilute acetic acid, and a second precipitation with one-third the volume of alcohol. Avoidance of the use of strong alcohol and of drying the material precluded the formation of a water-insoluble component, which could, however, be rendered soluble again by taking up in dilute base, acidifying slightly, and precipitating in 30% alcohol without now permitting the substance to dry out. The final method of isolation adopted therefore avoided the use of alcohol stronger than 30% (final concentration); furthermore, neither tissues nor pigment were dried unless this was necessary for analysis. The final dry weight was determined by evaporating aliquot portions of the pigment solution to dryness at 80° to 90°C ., and the ash values were readily obtained from such residues.

Table I furnishes representative data regarding the proportional weights of the branchial hearts, and the yield of adenochrome stored therein. It will be noted that the glands themselves are very small, their wet weight constituting only from 0.13 to 0.47% of that of the whole body, the average value being about 0.23%. There appears to be no correlation with age (weight) or sex. The presence of blood in the removed glands could hardly have introduced a variation greater than about 0.06 g., but this possible factor has not been seriously considered, since the isolated glands often continued to pulsate without visible bleeding.

Taking the average dry weight as 26.5% for grouped specimens Nos. 6 to 9 and employing the *final* weight of recovered adenochrome

TABLE I
Proportional Weights of Branchial Hearts; Yields of Pigment

Octopus No (sex)	Wet body wt.	Wet wt. of branchial hearts (% of total)	Dry wt. of branchial hearts (% dry wt.)	Wt. pigment, 1st pptn. (% yielded)	Wt. pigment, 2nd pptn. (% yielded)
	g.	g.	g.	g.	g.
1 (♂)	791	2.343 (0.29%)	0.712 (30.3%)	0.300 (42.1%)	0.263 (37.0%)
2 (♀)	1116.5	3.29 (0.295%)	0.91 (27.6%)	0.250 (27.4%)	0.196 (21.6%)
3 (♂)	103.7	0.244 (0.235%)			
4 (♀)	288.0	0.495 (0.17%)	0.126 (25.4%)	0.018 (14.3%)	0.014* (11.1%)
5 (♀)	382.6	0.494 (0.13%)	0.132 (26.6%)	0.022 (16.7%)	
6 (♂)	348.6	0.519 (0.15%)			
7 (♂)	263.0	0.554 (0.21%)			0.071 (3.28% of wet, or ca. 12.4% of dry)
8 (♀)	272.5	0.382 (0.14%)			
9 (♀)	489.0	0.706 (0.14%)			
10 (♂)	189.2	0.885 (0.47%)			

* Low figure; slight loss of pigment occurred.

(after a second precipitation, save in the case of specimen No. 5, wherein this step was omitted), we arrived at a figure of 23% as the approximate mean yield of pigment from dry gland, the extremes being 11% and 37%.

C. *Spectrum and chemical properties*: Adenochrome, prepared in the dry state, is an amorphous mauve or purple powder with slightly hygroscopic properties. It shows no visible evidence of decomposition at temperatures up to 300°C., and has no melting point, but chars readily at higher temperatures.

Besides its insolubility in alcohol or acetone, and its precipitation thereby from neutral or acidified aqueous solutions, adenochrome fails to dissolve in ether, chloroform, dioxane, glacial acetic acid, and most other organic solvents. It was found to be soluble in pure fused trichloroacetic acid, diluted pyridine, and slightly in hot glycerol.

Aqueous solutions of the pigment showed relatively low tinctorial power. Therefore, in order to observe the absorption spectrum, care was exercised to obtain a relatively strong aqueous solution, free from excessive optical scattering. Although a minor Tyndall beam was always present, dilute NH_4OH or Na_2CO_3 served to minimize scattering which otherwise obliterated the broad maximum observed between about 500 to 520 $\text{m}\mu$.

A Bausch and Lomb visual spectrophotometer was used in direct observation of the absorption maximum of adenochrome in dilute alkali. In Curve A, Fig. 2, the extinction coefficient e is plotted against wave length of emergent light. The formula is employed as follows:

$$e = \frac{\log_{10} \frac{I_0}{I}}{cL}$$

where $\log_{10} \frac{I_0}{I} = d$ or Density units measured directly by the instrument.

- I_0 = The light transmitted by solvent alone, and
- I = That transmitted by the pigment system,
- c = Concentration of pigment in grams per liter,
- L = Length of column of solution in centimeters.

In curve B, d of a solution of pigment in a relatively high state of purification but of unknown concentration is plotted against wave length. The data serve to confirm the shape of curve A, and to sharpen the region of maximum absorption in the broad, smooth region involved. Our pigment shows a maximum at about 505 $\text{m}\mu$.

The color of the pigment itself, and color-changes produced by reactions with certain reagents were useful in studying some further properties of adenochrome. Following are some of its reactions.

*Oxidizing agents*Concentrated HNO_3 :

Bright orange color.

Dilute KMnO_4 :

Brown red, bleaching to brown yellow (unlike yellow of urochrome so treated).

Withdrawal of O_2 :

No change.

Concentrated H_2SO_4 :

Brown color, deepening by charring.

Bromine:

Dark amorphous precipitate.

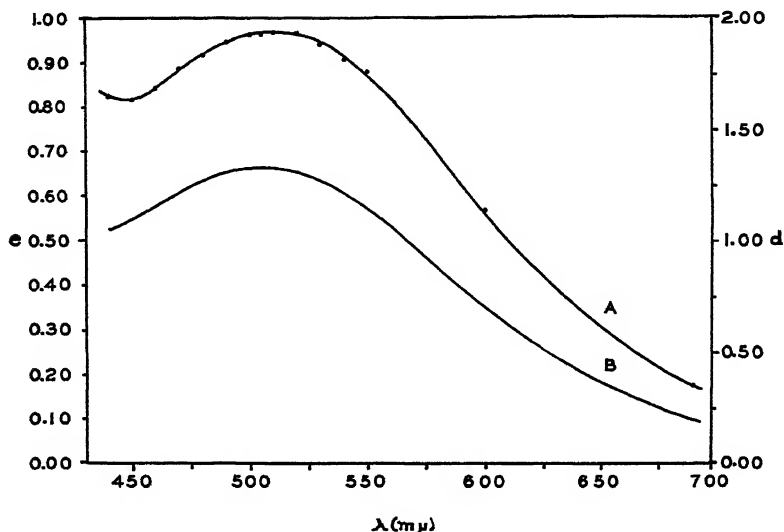


FIG. 2. Absorption spectrum of adenochrome in water containing traces of alkali

Curve A: Wave length plotted against absorption coefficient, e (left).

Curve B: Wave length plotted against density, d (right).

*Reducing agents*Zinc dust + KOH :

Rapid decolorization.

 Na_2S :

No immediate reaction, but boiling changed pink color to pale brown.

 $\text{Na}_2\text{S}_2\text{O}_4$:

Bleached to pale yellow color, reversible by aeration.

 NaHSO_3 :

No change.

Adsorbents

Talc:

No adsorption.

Kaolin:

No adsorption.

 Al_2O_3 :

No adsorption.

Starch:

No adsorption.

 MgO :

Strong adsorption

 $\text{Ca}(\text{OH})_2$:

Strong adsorption

Probably because of pigments' acidic properties, hence its ability to form salts.

Specific tests

Salkowski's, for indole, pyrrole:	Negative, with raw pigment or hydrolyzates.
Pine-splinter + HCl for pyrrole:	Positive, from roasting pigment.
Murexide test for purines:	Negative.
Schiff's, for aldehydes:	Negative.
Liebermann's, for monohydroxy phenols:	Negative.
Benedict's for reducing sugars:	Negative on acid hydrolyzate (no carbohydrate material).
Lipoids:	Absent, shown by insolubility in ordinary lipid solvents, solubility in aqueous systems.
Phosphorus:	Absent, shown by negative reaction with molybdate + oxidized digest.
Halogens:	Absent, shown by negative reaction with AgNO_3 + digest.

Protein reactions. A (adenochrome similar)

Roasting:	Familiar odor of burning hair or feathers.
Ninhydrin reaction:	Strong (α -amino groups, or certain alkyl amines).
Biuret reaction:	Very faint.
Diffusion through animal membranes:	No.
Precipitation with dialyzed iron:	Quantitative.
Precipitation with FeCl_3 :	Yes.
Precipitation with Ag^+ :	Yes.
Precipitation with Cu^{++} :	Yes.
Precipitation with Pb^{++} :	Yes.
Precipitation with Hg^{++} :	Yes.
Precipitation with phosphotungstate:	Yes.
Precipitation with phosphomolybdate:	Yes.
Precipitation with picrate:	Yes.

Protein reactions. B (adenochrome dissimilar)

Boiling water or dilute acid:	No coagulum.
Alcohol or acetone:	Precipitate redissolved readily in dilute alkali.
Xanthoproteic reaction:	Negative.
Millon's test:	Negative.
Aldehyde test for tryptophan:	Negative.
Sakaguchi reaction for arginine:	Negative.
Reduced sulfur:	Absent (all S in oxidized state).
Trichloroacetic acid:	Dissolved powdered pigment (precipitates proteins).

The alkaline and the acid hydrolyzates of adenochrome failed to show any of the principal reactions not manifested by the original pigment.

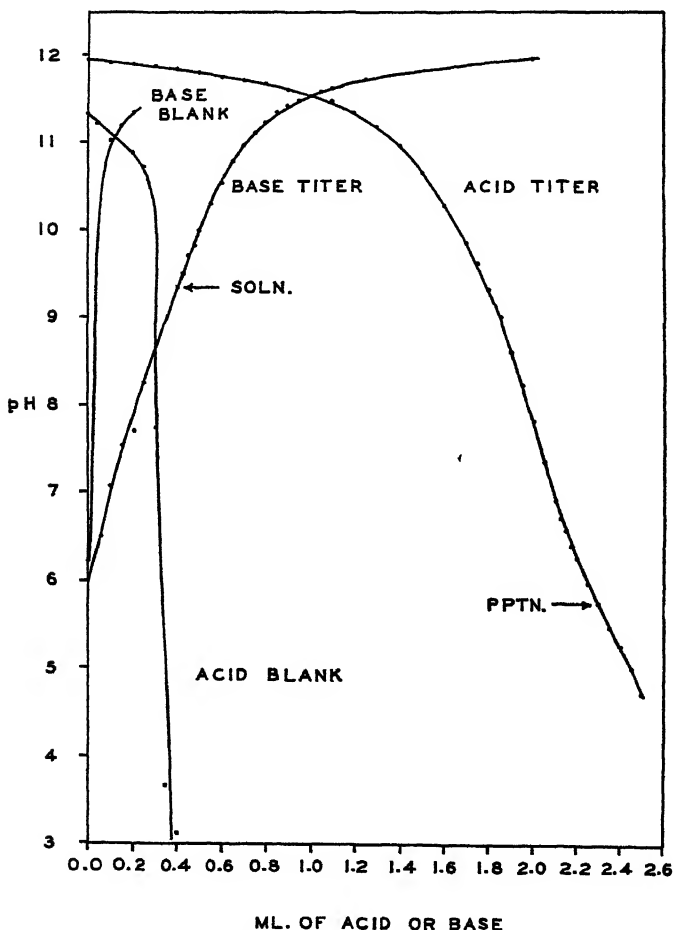


FIG. 3. Characteristic titration curves for adenochrome

Initial concentrations: 0.0775 g. adenochrome, partly dissolved, partly suspended in 10 ml. H_2O ; titrated directly with 0.306 N NaOH to pH 11.95; back titrated with 0.26 N HCl , using a Beckmann glass electrode and pH meter.

Although adenochrome bears a few resemblances to protein, certain striking dissimilarities exclude it from identification with that class of compounds.

Attempts were made to gain an idea of the equivalent weight of adenochrome by titrating it with standard alkali, using the Beckmann glass electrode and pH meter calibrated with the use of a standard solution of *d*-glutamic acid. Fig. 3 shows that, while adenochrome possesses considerable buffering capacity as a weak acid, its titration curve is quite smooth throughout, the absence of a break making it impossible to determine the true point of neutralization. Our adenochrome-in-water system showed an initial pH value of about 5.97. The particulate phase disappeared at about pH 9.35, giving way to the familiar colloidal condition. During back-titration with standard acid, wherein a smooth curve of similar shape resulted, reprecipitation of the pigment as red-purple flocks took place near pH 5.75.

The low solubility of our product, and the resulting necessity of working with very dilute solutions (save when in the distinctly alkaline phase), are contributing factors to the difficulty of establishing a range of neutralization in titration curves. The initial slightly acidic condition of adenochrome in water, and the gradual increase in pH with added alkali, in contrast to the rapid rise shown by the control, would seem to characterize the pigment as a compound containing a number of weakly acidic groups. Since phenols are absent, these acidic groups are probably carboxyl, perhaps accompanied by one or more sulfoxyl bodies per molecule.

ELEMENTARY COMPOSITION

Qualitative analytical tests showed the presence of C, H, N, S (oxidized state) and ash containing Fe. Through the kindness of Dr. A. J. Haagen-Smit at the California Institute of Technology, two different preparations of adenochrome were dried in high vacuum at 50°C. for 30 minutes and subjected to quantitative microanalysis for C, H, N and ash, and one sample was analyzed for S. The pigment's insolubility in camphor precluded a molecular weight determination.

Carefully and repeatedly purified preparations of pigment samples showed considerable variation in ash (*i.e.*, from 4.2 to 7.4%) and in total iron (0.43 to 0.67%). Iron was determined either in ashed samples by the colorimetric method of Wong (8) or without preliminary ashing by a slight modification of his method, giving identical results by both procedures.

Iron seems to be present chiefly in the ferric state, from which it is reduced by the pigment in acidic systems. It is possible that we may be dealing with a ferric compound associated with the larger ade-

nochrome molecule through a partial neutralization of colloidal charges. In this connection dialyzed iron, a colloid carrying a positive charge, is

TABLE II

Elementary Micro-analysis of Adenochrome (Kindness of Dr. A. J. Haagen-Smit and Dr. G. Oppenheimer)

	mg. sample	mg. CO ₂	% C	mg. H ₂ O	% H
Preparation A					
Sample 1	3.833	5.514	39.26	1.827	5.33
Sample 2	3.035	4.505	40.51	1.446	5.33
		ml. N ₂	% N		
Sample 1 ..	3.603	0.423 (27°C.; 744 mm.)	12.78		
Sample 2	2.339	0.243 (26°C.; 744 mm.)	11.33		
		mg. ash	% ash		
Sample 1....	3.833	0.188	4.90		
Sample 2	3.035	0.152	5.00		
		mg. CO ₂	% C		
Preparation B					
Sample 1....	3.205	4.428	37.70	1.663	5.81
Sample 2..	2.532	3.496	37.68	1.352	5.97
		ml. N ₂	% N		
Sample 1..	3.090	0.392 (26°C.; 741 mm.)	13.78		
Sample 2..	2.876	0.368 (25°C.; 741 mm.)	13.93		
		mg. ash	% ash	mg. BaSO ₄	% S
Sample 1.....	4.233	0.308	7.28	1.664	5.40
Sample 2	5.687	0.423	7.44	2.172	5.24

readily adsorbed to adenochrome and brings about mutual precipitation if added in sufficient quantity. Ferric chloride has a like effect.

Iron constitutes but a minute portion of the pigment, and only from

one tenth to one eighth of the ash, in which were detected no alkali metals, alkaline earths nor any traces of copper (by diethyl-dithiocarbamate test). Most other heavy metals were also ruled out by the failure of H_2S to yield precipitates in iron-free acidic solutions of the ash. However, a faint dark turbidity appeared in iron-free ammoniacal solutions of the ash in the presence of H_2S . This suggested possible traces of nickel or cobalt, but our small supply of material did not allow further investigation of these trace-metals.

Because iron was present in only minute quantities, inconstant in relative amounts, easily and quantitatively split away from the pigment

TABLE III
Composition of Adenochrome

	1. (Average of microanalyses)	2. (Ash-free basis)	3. (Per cent com- position/atomic weight)	4. (Data of column 3, multiplied by 12, as least common multiple to give approximate whole numbers)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
C	38.79	41.33	3.44	41.33
H	5.61	5.98	5.98	71.76
N	12.955	13.80	0.985	11.82
S	5.32	5.67	0.177	2.12
O	31.17*	33.22	2.076	24.91
Ash	6.155			
	100.000	100.00		

* By difference.

and representing only a small fraction of the total ash, we concluded that it may be associated with the adenochrome molecule only in an incidental way, and not stoichiometrically. The change in color of the pigment from red or pink to pale blue or green by the addition of acids was reversed by alkali, although the traces of iron were dissociated from the pigment by treatment with acids, and probably remained so.

Table II shows figures which yield grand average values for the elementary composition of adenochrome shown in Table III (Column 1). In the light of the above considerations regarding the disposition of the small amounts of iron, we recalculated the composition of the pigment on an ash-free basis, obtaining the results shown in Column 2. Column

3 shows the quotient of the percentage of each element divided by its atomic weight. In order to arrive at a provisional empirical formula for adenochrome, it is necessary to show at least one atom of sulfur. Multiplying the values in Column 3 by 6, however, while giving 1.06 atom of sulfur, yields fractional values for those of carbon (20.66) and oxygen (12.45). We have therefore used the next possible multiplier, 12, with results shown in Column 4 which are probably better approximations to the real values.

While the calculated numbers of constituent atoms do not emerge as whole numbers, there is seen to be at least a fair degree of approximation. Furthermore, our observations and the microanalytical results would indicate that adenochrome is not a substance which is easy, or even possible to prepare in absolutely pure form, just as the complex urinary pigment urochrome and the widely distributed black pigment melanin have never been so prepared. Our provisional empirical formula for adenochrome, based on the above data, would appear to be approximately according to the constitution $C_{41}H_{72}N_{12}O_{25}S_2$, with a minimum molecular weight of about 1200.

STATE OF SULFUR

On numerous occasions isolated preparations of the pigment were treated with boiling alkali, and the digest subsequently tested for reduced sulfur with lead acetate. Even after prolonged treatment with concentrated NaOH, no traces of PbS were ever obtained; thus the possible presence of cystine, or of any other compounds containing —S—S— or —SH groups were eliminated. Prolonged boiling in concentrated alkali or fusion in $Na_2CO_3 + KNO_3$ mixture yielded SO_4^{--} , however, precipitable as the barium salt which could be dissolved in concentrated H_2SO_4 and reprecipitated by dilution. Although sulfur was thus demonstrated to be present in oxidized form, its position in the pigment was not determined. Maintaining the pigment in the presence of $Ba(NO_3)_2$ in neutral, acidic, or alkaline solution for several hours at $100^\circ C.$ failed to bring about the precipitation of $BaSO_4$, although the pigment underwent some decomposition or precipitation. This would indicate that the oxidized sulphur was held in a stable position.

QUALITATIVE DISTRIBUTION OF NITROGEN

Solutions of adenochrome so dilute as to show no color alone gave a blue violet color with triketohydrindene hydrate. Use was made of the

adaptation of this color reaction by Harding and MacLean (9) for the determination of α -amino nitrogen, although the reaction is also given by ethyl amine, trimethyl amine and benzyl amine.

Instead of comparing our solutions directly with a standard reference compound in a colorimeter, we measured the light absorption of solutions at $571\text{ m}\mu$, the absorption maximum, with the use of the Bausch and Lomb visual spectrophotometer. For comparison, a standard solution of Eastman's *d,l*-alanine was used. By this method adenochrome was estimated to contain quantities of α -amino nitrogen (or alkyl amines) corresponding to 2.88%.

Effervescence of very small bubbles was observed when adenochrome (even when prepared by extraction with 1% Na_2CO_3 instead of dilute ammonia) was treated with cold concentrated NaOH or KOH . Moist litmus paper suspended above such systems soon turned blue in color. Accordingly, amide or ammonium nitrogen was suspected. A small quantity (0.0692 g.) of the pigment was treated with ca. 8% KOH (initial concentration) in a Kjeldahl flask, and the contents were boiled gently, the distillate being collected in dilute standardized HCl . When volatile base ceased to appear in the distillate, aliquot samples were estimated for ammonia by nesslerization, using a standard $(\text{NH}_4)_2\text{SO}_4$ solution for comparison. Aliquots were also titrated with dilute standardized NaOH using methyl orange as indicator.

A slight loss of distillate doubtless rendered somewhat low the nesslerization figure of 4%, and gave a high value to the titration figure of 7.7%. However, the apparent amide nitrogen value lay between these limits and was assumed to represent ammonia, although we might have been dealing with alkyl amines or with mixtures of volatile bases.

Following the distillation of all volatile base, further boiling with the now very concentrated KOH caused the distillation of traces of a non-basic substance of very foul odor, reminiscent of fecal compounds. It gave no test for indole or pyrrole with *p*-dimethylaminobenzaldehyde, but the addition of phenyl diazonium sulfonate gave a weakly positive reaction indicative of imidazoles.

Fresh adenochrome solutions, as well as the distillation-residue from the above experiment gave consistently negative results with Millon's test for phenols; the phenol test with FeCl_3 was inconclusive. Positive reactions for the imidazole nucleus were always obtained with the Ehrlich diazo reagent (sulfanilic acid in dilute HCl or HNO_3 ; dilute NaNO_2). The yellow color which finally developed from an initial

orange was similar to that of histidine treated in like manner, which goes through a preliminary red phase.

Therefore, the procedure described by Snell (10; pp. 219 *et seq.*) was employed, using aliquot portions of the adenochrome distillation-residue and a standardized dilute solution of histidine, each diazotized and matched spectrophotometrically at 440 $m\mu$ (the maximum point of absorption). Our results indicated that roughly 2 to 3% of the pigment existed as imidazole nitrogen. The imidazole configuration in adenochrome was concluded not to be histidine or histamine, since neither of these compounds yielded a substance in their distillates which gave a positive reaction with Ehrlich's reagent.

Chemical comparisons show some resemblances and some striking differences between adenochrome and urochrome, the yellow urinary pigment of mammals.

The extensive crustacean factor in the normal diet of the octopus suggested also a comparison of adenochrome with Verne's amino acid pigment from the hypodermis of crustaceans.

Table IV presents a resumé of such properties of adenochrome as render possible a comparison with urochrome, described by Verne (11) and Dombrowski (12), and with Verne's crustacean pigment (13).

The comparison, while revealing the numerous differences between adenochrome and urochrome, brings out a sufficient number of similarities to suggest the possibility of some metabolic parallelism in the elaboration of the two pigments in widely separated phyla. The known properties of Verne's crustacean pigment are too few for an extensive comparison, but the substance is clearly different from adenochrome. Verne reports positive xanthoproteic and Millon's reactions with his crustacean pigment, but is not certain that a protein contaminant might not be responsible.

Adenochrome occupies a rather unique position from the standpoint of both biochemistry and physiology. Our studies would suggest that the pigment is a nitrogenous waste product. This is indicated especially by the apparent storage of much nitrogen in an amide state, releasable as free ammonia in the presence of strong alkali; the pigment thus may act as a kind of reservoir to maintain catabolic ammonia and/or perhaps alkyl amines (giving the ninhydrin reaction), beneath the toxic threshold. Again, the presence of considerable imidazole nitrogen suggests that we may be dealing with an excretory substance analogous to the waste product, urochrome. The presence of oxidized

TABLE IV

Comparison Between Adenochrome, Urochrome, and Verne's Amino Acid Pigment

	Adenochrome	Urochrome	Verne's amino acid pigment
1. Source	Branchial hearts of octopus	Urine of mammals (increased in fever, etc.)	Chromatophores of crustacean hypodermis.
2. Color	Red in solution; amorphous mauve to purple powder when dry.	Yellow in solution; amorphous brown powder when dry.	Yellow.
3. Composition	C 41.33% H 5.98 N 13.80 S 5.67 O 33.22 (Fe traces)	C 43.42% H 5.32 N 10.78 S 5.9 O 34.58	—
4 Solubilities	Sl. sol. H ₂ O; v. sl. sol. dil. acids; v. sol. alkalis; sol. strong acids; sl. sol. trichloroacetic acid; sol. dil. pyridine; insol. 30 to 100% alc., acetone, ether, chloroform, glacial acetic a., CS ₂ , pure pyridine, cold glycerol. Slightly hygroscopic.	V. sol. H ₂ O; sol. acids and alkalis easily altered thereby; sl. sol. alc.; insol. ether, benzene, chloroform. Very hygroscopic.	Sol. cold H ₂ O; v. sol. hot H ₂ O; sol. dil. acids and bases, trichloroacetic acid; insol. alc. and fat solvents.
5. Reduced sulfur	Absent; all S in oxidized form.	Present.	Present.
6. Pauly's diazo reaction	Positive.	Positive.	—
7. Pyrrole test	Positive.	Positive.	—
8. Murexide test	Negative.	—	Negative.

TABLE IV—*Concluded*

	Adenochrome	Urochrome	Verne's amino acid pigment
9. Acid hydrolysis	6 to 8 hrs. with 33% H_2SO_4 gave little brown sediment; principal chemical reactions unchanged.	8 hrs. with ca. 6 <i>N</i> HCl gave uromelanin quantitatively.	—
10. Free NH_3	Readily evolved.	—	Not detected.
11. Protein precipitants	Pptd. by Ag^+ in alk. soln.; Cu^{++} in neut. soln.; Pb^{++} , Fe^{+++} in aq. soln's, and Hg^{++} in acid or neut. soln.; by phosphotungstic, phosphomolybdic or picric acids.	Pptd. from aq. soln's by Cu^{++} , Hg^{++} , Pb^{++} , or Fe^{+++} ; also by phosphotungstic or phosphomolybdic acid.	Not pptd. by ammoniacal AgNO_3 or by CuSO_4 after treatment with NaHSO_3 .
12. Weiss or Hefebower test for urochrome or urochromogen	Dil. $\text{KMnO}_4 \rightarrow$ brownish color.	Dil. $\text{KMnO}_4 \rightarrow$ increased yellow color.	—
13. Thudichum's test for urochrome.	$\text{Hg}(\text{NO}_3)_2 \rightarrow$ red-brown pptn., unchanged by boiling.	$\text{Hg}(\text{NO}_3)_2 \rightarrow$ white pptn., boiling \rightarrow rose red.	—

sulfur in the pigment may be indicative of the operation of detoxicating mechanisms similar to those which take place in mammalian tissues, involving the sulfonation of certain poisonous molecules.

The pigment is stored solely in the glandular tissue of the two branchial hearts, whose major blood supply has first passed through that region of the vena cava which is invested with the excretory nephridial gland. Should the nephridial gland constitute a channel for the gradual excretion of adenochrome, the presence of this pigment in the gland would have been expected, but such was never observed to be the case.

Nor was any evidence found for the possible elimination of the pigment from the gill tissues. Indeed any efficient manner of elimination of the pigment was difficult to imagine, since adenochrome is an acidic substance, soluble in faintly alkaline media, whereas the pH of fresh branchial heart tissue was from 4.9 to 5.04 (Beckmann glass electrode) and hence afforded an unfavorable medium for the pigment's dissolution or hence its transportation.

We have not been able to decide upon the metabolic origin or fate of adenochrome. It is hoped that future investigations may be more revealing in this regard.

Acknowledgments: We take pleasure in expressing our appreciation and thanks to the following Dr. Wesley R. Coe, Professor Emeritus of Zoology, Yale University, for his friendly interest, useful advice and generous assistance; Dr. A. J. Haagen-Smit, in charge of the microanalytical laboratory at the California Institute of Technology, and Dr. G. Oppenheimer, microanalyst in the same laboratory, for the elementary analyses of our material, and Professor L. Zechmeister at the California Institute of Technology for his continued friendly interest.

SUMMARY

1. The glandular walls of the gill-hearts in the octopus *Paroctopus bimaculatus* contain relatively large quantities of a red, intracellular acidic pigment, adenochrome, which is readily extracted with dilute alkalis to give wine-red colloidal solutions with a single absorption-maximum in the visible spectrum at about 505 m μ .

2. Adenochrome is insoluble in ordinary fat-solvents including alcohol and acetone, either of which precipitate it from neutral or slightly acidic aqueous systems as amorphous flocks, readily resolvable in dilute alkali. The purified pigment shows no melting point, but decomposes above 300°.

3. Readily oxidized by concentrated nitric or sulfuric acids, KMnO₄, or bromine, adenochrome is also readily reduced by zinc dust and alkali, warm Na₂S, and reversibly by hydrosulfite.

4. The new pigment fails to show several critical reactions of proteins, and exhibits some types of behavior not encountered in proteins. It was estimated to contain nearly 3% of α -amino nitrogen and/or alkyl amines, some 4 to 7% of amide nitrogen, and comparable amounts of imidazole nitrogen. Pyrrole bodies were demonstrable only by roasting. Neither purines nor phenols were detected.

5. The ash content varied between about 4 and 7.4%, invariably

containing traces of iron, besides possibly cobalt or nickel, but no copper, alkali metals or alkaline earths. The iron seemed to be present only incidentally.

6. From microanalytical data, the minimum empirical formula for adenochrome has been calculated on an ash-free basis as $C_{41}H_{72}N_{12}O_{25}S_2$, with a minimum molecular weight of about 1200.

7. The elementary constitution of adenochrome is similar to that of urochrome, but the two compounds differ in many respects, notably in disposition of sulfur which is entirely in the oxidized condition in adenochrome, but yielded in the reduced state by urochrome.

8. The possible position of adenochrome as an excretory substance is discussed briefly.

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Changes in Properties of Protyrosinase Due to Shaking*

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Since the properties of soluble proteins are generally known to be changed by spreading as a monolayer, it was thought advisable to study such an effect on protyrosinase. The following results have been obtained by the use of a shaking method.

METHODS

Grasshopper egg protyrosinase was extracted by following a described method (1). To glass vials of 9.0 ml. capacity were added 2.5 ml. of 0.9 per cent NaCl, 1.25 ml. of 0.2 *M* phosphate buffer (pH 6.8), and 0.5 ml. of protyrosinase. If greater amounts of protyrosinase were to be tested, correspondingly lesser amounts of NaCl solution were added. Saponin (Merck) and sodium dodecyl sulfate (duPont) were dissolved and added with the NaCl solution. Octanol and dodecanol (Eastman) were added from petrol ether solutions. After the ether had evaporated, the solutions to be tested were added. The vials were stoppered with screw caps and squares of either Cellophane or Parafilm. Prepared vials were then fastened with the long axes across the arm of a Leeds and Northrup hydrogen electrode shaker which oscillated through an arc subtended by an angle of 38 degrees. The rate of shaking was varied with a rheostat and was held constant by an automatically regulated AC current source. Shaking was done either at room or refrigerator temperatures.

Aliquots from the vials were analyzed for protyrosinase, tyrosinase, and inactive decomposition products. These analyses were performed with a Warburg apparatus as previously described (2) by measuring the velocity of oxidation of tyramine hydrochloride. The reaction velocity for that analysis of the control or the unshaken vial's contents in

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the presence of excess activator (sodium dodecyl sulfate) was given the value of unity. Various shaken portions of the same extract were compared on the basis of the relation which their reaction velocities bore to that of the control.

RESULTS

The shaking treatment seems to produce a number of changes. Of the original, soluble protyrosinase 0.35 changes into tyrosinase. Inactive products appear to the extent of 0.39, which value was derived from the difference in the sum of protyrosinase and tyrosinase occurring at the beginning and at the end of the shaking period. A visible, shreddy precipitate is also produced. When this precipitate was centrifuged down and tested, it was found as shaking proceeded that protyrosinase and tyrosinase left the supernatant and entered the precipitate. These various changes were analyzed in order to see if a first order reaction was followed. When values of the initial or ultimate concentrations and amounts reacting at any time are introduced into the first order equation, four curves yielding approximations to experimental values are then determined (Fig. 1). Some useful information is thus found. For example, while protyrosinase decreases at the rate of 0.15 per cent per second, inactive products and tyrosinase appear at 0.18 and 0.12 per second. Since protyrosinase and tyrosinase are precipitated at a velocity identical to that of the formation of inactive products, it seems that these two processes in some way are associated. The data in Fig. 1 were obtained from experiments in which the initial concentration of protyrosinase in terms of non-dialyzable material was approximately 0.06 per cent. Other shaking experiments were performed in which the protyrosinase concentration was 0.12, 0.24, and 0.36 per cent. For any of the latter, the velocity constants were identical with those for 0.06 per cent protyrosinase. In other words, all other conditions being constant, the half-life period of protyrosinase is 462 seconds throughout a six-fold range in concentration. Beyond 50 minutes, shaking can proceed up to 24 hours with no appreciable changes in the composition of the mixtures. Once the changes due to shaking have been completed, no reversion in the mixtures has been detected for so long as some 24 hours at room or refrigerator temperature.

Variation in rate of shaking at either room or refrigerator temperature has no influence on the ultimate composition of the mixtures. However, considerable effects on the rates of change were noted (Fig. 2). Since

the slopes of the lines through the velocity constants at various rates of shaking are identical, it seems that production of tyrosinase is independent of temperature yet dependent on the rate of shaking. Production of inactive products seems dependent on both the rate of shaking and the temperature. The rate of decomposition of protyrosinase,

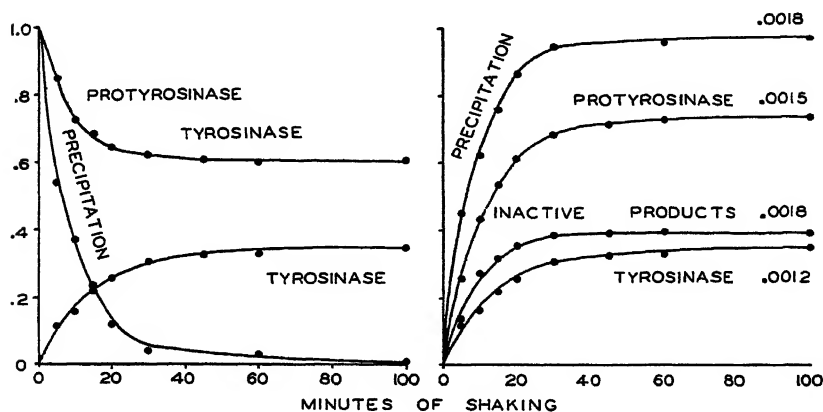


FIG. 1. The Time Course of Change in a Solution of Protyrosinase Due to Shaking at 2.0 Oscillations per Second at Room Temperature

The ordinates are the ratios of the velocity of enzymic action from contents of vials which had been shaken to that which had not been shaken and to which during analysis excess activator, sodium dodecyl sulfate, had been added. The points of the "tyrosinase" curve were obtained from analyses performed in the absence of sodium dodecyl sulfate. Those of the "protyrosinase and tyrosinase" curve are from analyses performed in the presence of excess sodium dodecyl sulfate. Similar analyses were performed on the supernatant resulting from centrifuging 20 minutes at 1800 G's in an angle centrifuge. The curves are constructed from the first order equation;

$$k = (2.3/t) (\log a/a - x),$$

where t = time in seconds of shaking, a = greatest amount reacting and x = amount reacting in time t .

being a function of the rate of production of tyrosinase and inactive products, also proved dependent on temperature and rate of shaking. When shaking was less than 0.5 per second there was no detectable effect on protyrosinase. It seems that a critical rate of shaking exists. Changes proceed more rapidly above this critical value.

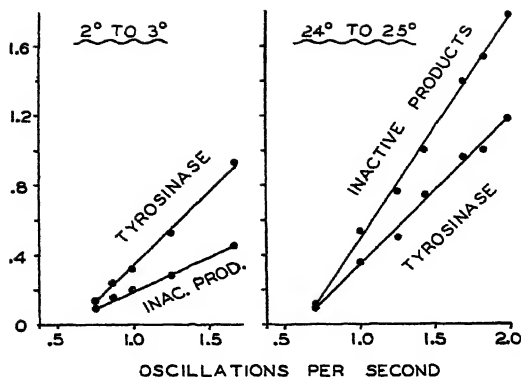


FIG. 2. The Effect of Variation in Rate of Shaking at Different Temperatures on the Velocity of Production of Tyrosinase and Inactive Products
The ordinates are first order specific reaction rates multiplied by 10^3 .

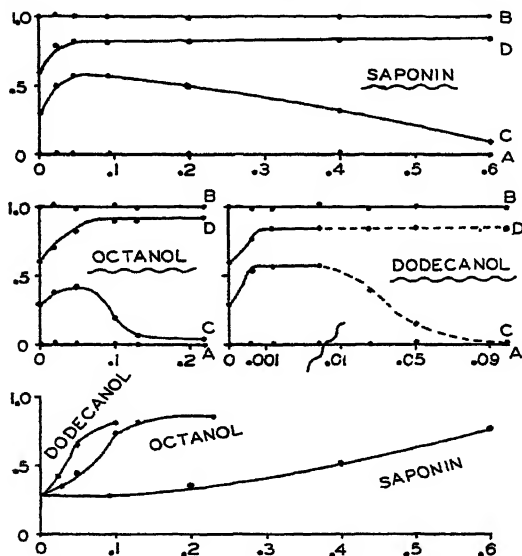


FIG. 3. Upper and Middle Panels. The Effect of Surface-Active Non-Electrolytes on the Composition of Mixtures of Protyrosinase, Tyrosinase, and Inactive Products Remaining After 1 Hour of Shaking at 2.0 Oscillations per Second at Room Temperature

The ordinates are similar to those of Fig. 1. The abscissae are concentrations in per cent. Curves A and B without shaking. Curves C and D with shaking. Curves B and D are from analyses performed with added excess sodium dodecyl sulfate whereas curves A and C are from analyses done in the absence of sodium dodecyl sulfate.

Lower panel. Relative amount of protyrosinase remaining after shaking.

Surface-active non-electrolytes, such as saponin, octanol, and dodecanol, in various concentrations were added. The effect of these compounds is illustrated in Fig. 3. The upper panel line A, coinciding with the abscissa, shows that increasing concentrations of saponin do not change protyrosinase into tyrosinase. The position and parallel course of line B, since all the protyrosinase can be recovered, shows that saponin itself does not destroy protyrosinase. Both these sets of data (A and B, Fig. 3) are from quiescent solutions. The two intermediate lines, C and D, are results from shaking experiments. Of these two lines the lower, C, shows the relative amount of tyrosinase produced by shaking, whereas the upper, D, indicates the sum of protyrosinase and tyrosinase remaining after shaking. The difference between these two, which is graphically illustrated in the lower panel of Fig. 3, pertains to the relative amount of protyrosinase remaining. It also follows that the difference between the two upper lines, B and D, indicates the amount of destruction or formation of inactive products. For example in the absence of saponin, tyrosinase, protyrosinase, and inactive products occur as 0.30, 0.30, and 0.40 respectively. In 0.05 per cent saponin the occurrence changes toward 0.60 tyrosinase, 0.25 protyrosinase, and 0.15 inactive products. With increasing concentrations of saponin inactive products remain at 0.15, whereas tyrosinase decreases to 0.10 and protyrosinase accordingly changes to 0.75. Effects of octanol and dodecanol (Fig. 3, middle panel) resemble those of saponin except in so far as concentration differences are concerned.

Sodium dodecyl sulfate, which dissociates to give a surface-active anion, is an activator of protyrosinase (1). The activation function of sodium dodecyl sulfate is illustrated by the "quiescent" curve in Fig. 4. In contrast to shaking effects the dodecyl sulfate shows no formation of inactive products, *i.e.*, protyrosinase is entirely converted into tyrosinase. If mixtures which have been partially or completely activated by dodecyl sulfate are shaken, inactivation then occurs. This phenom-

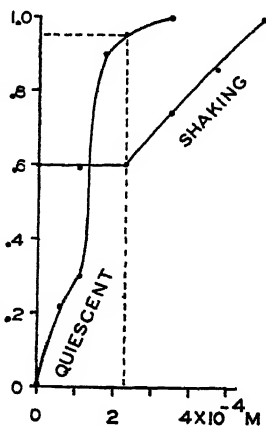


FIG. 4 The Activation of Prottyrosinase by a Surface-Active Anion, Dodecyl Sulfate, and the Result of 1 Hour Shaking on an Initial Mixture of Prottyrosinase and This Activator

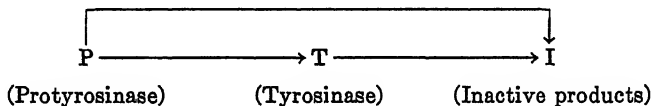
Ordinates, same as those of Fig. 1. Abscissa, molar concentration of sodium dodecyl sulfate.

In contrast to shaking effects the dodecyl sulfate shows no formation of inactive products, *i.e.*, protyrosinase is entirely converted into tyrosinase. If mixtures which have been partially or completely activated by dodecyl sulfate are shaken, inactivation then occurs. This phenom-

non is illustrated by the "shaking" curve of Fig. 4. Even when enough dodecyl sulfate is present to form 0.95 tyrosinase, it is evident that with shaking, this mixture in time is 0.40 destroyed, *i.e.*, tyrosinase, however produced, when shaken yields inactive products. In 0.0006 *M* dodecyl sulfate there seems to be no destruction of tyrosinase indicating a secondary, protective (?) action.

DISCUSSION

The following statements are summary, yet they are also intended to be by nature introductory to a discussion. As a result of shaking, a solution of protyrosinase changes irreversibly at characteristic velocities and order into definite proportions of protyrosinase, tyrosinase, and inactive products. Destruction as well as activation occur. Even with prolonged shaking some protyrosinase remains. Perhaps these changes proceed as a branching, consecutive reaction:



If inactive products are insoluble and capable of combining with both protyrosinase and tyrosinase, then the reactions halt, because protyrosinase and tyrosinase are no longer dispersed.

No reasons as yet have been found for not considering protyrosinase as being in part at least if not entirely, a protein. Since various proteins as a result of shaking, become insoluble (3, 4), or acquire thiol groups (5) it is not surprising to find that protyrosinase also is changed by similar treatment. What seems of interest, however, with respect to shaking of protyrosinase solutions, is the fact that two kinds of products are formed. One of these products is catalytically active, whereas the other is inactive. If denaturation is defined in terms of solubility or thiol groups, then formation of the catalytically active product is not an example of a classic denaturation. In experiments with protocols similar to these no formation of thiol groups during production of tyrosinase was found (6). Only after destruction of potentially active and active catalytic material was there an increase in thiol groups. One may, therefore, infer that inactive products are insoluble and contain numerous exposed thiol groups. During shaking these inactive products are produced and protyrosinase and tyrosinase, although ordinarily soluble, are precipitated at the same rate of 0.18 per cent per second. It seems

that unit production of insoluble, inactive products leads to unit combination with protyrosinase and tyrosinase. Such a process may in the end explain how protyrosinase during prolonged shaking is but partially rather than completely decomposed.

The branching, consecutive nature of the reactions produced by shaking may be ascertained from the data of Figs. 3 and 4 where it is shown that inactive products can derive from either protyrosinase or tyrosinase. When conditions are such that shaking produces little if any tyrosinase, inactive products can still be formed. Thus inactivation of protyrosinase can proceed without necessarily passing through tyrosinase. On the other hand tyrosinase under different conditions seems to yield inactive products.

Surface active compounds, saponin (3) and heptanol (4) in sufficient concentration have been found to inhibit surface denaturation of ovalbumin. This particular effect may be thought of as being due to continuous saturation of the surface. With each renewal of the surface a film of saponin may form at a rate in great excess of that for spreading and subsequent denaturation of the protein. These surface-active compounds have a similar but more complicated effect on the surface reactions of tyrosinase and protyrosinase. At certain concentrations dodecanol, octanol, and saponin inhibit the formation of tyrosinase to a greater extent than the formation of inactive products. In 0.0006 *M* sodium dodecyl sulfate there is no destruction of tyrosinase, whereas at lower concentrations destruction occurs. The pH stability of protyrosinase (2) is such that its activation has been compared with the splitting of other known copper-proteins into a greater number of lower molecular weight components (7). It is suggested that protyrosinase follows similar steps in the changes produced by shaking. A large, inactive, soluble molecule (protyrosinase) dissociates at a surface into smaller, active, soluble molecules (tyrosinase). Either of these spread as a monolayer of insoluble, inactivated protein which then removes the two previous molecules from solution.

SUMMARY

As a result of shaking above a critical rate, a solution of protyrosinase changes irreversibly at characteristic velocities and order into a mixture of definite proportions of protyrosinase, tyrosinase, and inactive products. Perhaps these changes proceed as a branching, consecutive reaction. If inactive products are insoluble and capable of combining

with both protyrosinase and tyrosinase, then the reactions halt, because neither of these are dispersed in solution and available for reaction at a surface.

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Constancy of Chemical Composition of Serum Proteins Regenerated on Various Dietary Regimes¹

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Several attempts have been made to alter the chemical composition of serum proteins by dietary means. Abderhalden (1) administered large amounts of the protein, gliadin, to a horse and to Eck-fistula dogs and determined the glutamic acid content of the protein of the cells and serum isolated from the blood of these animals. He found a normal glutamic acid content of the proteins. Lang (2) reported changes in the composition of human serum protein during a working day. Schenck and Kunstmann (3) observed variations in the content of various amino acids and amino nitrogen of the serum albumin and serum globulin of a normal human during a 24 hour fasting period. Ingestion of urea or protein also resulted in variations in the composition of these serum proteins. They concluded that serum proteins are not of constant composition and suggested that tissue proteins are converted into serum proteins and released into the blood stream. These findings could not be confirmed by Abderhalden (4) who demonstrated that the plasma proteins of the horse, cow, and rabbit which during four week periods were fed green food, bran, or oats, remained unchanged in amino acid composition. Dirr and co-workers have shown that after administration of arginine hydrochloride (5), tyrosine (6), or histidine hydrochloride (7), a striking increase in the serum protein content of the corresponding amino acid resulted. R. J. Block (8) observed no such increase when arginine was fed.

In view of these contradictory findings it seemed of value to determine the amino acid composition of the total serum protein of dogs during serum protein regeneration following the administration of various single

¹ Murrill, W. A., and Newburgh, L. H., *J. Biol. Chem.* **128**, lxxiv (1939).

proteins in the diets. Since it is impossible to prepare pure fractions of the serum proteins in which the sum of the pure fractions totals 100 per cent of the serum proteins, it was necessary to determine the amino acid content of the total serum proteins (an obvious mixture of proteins). Nevertheless, it was felt that the analysis of this mixture would more truly reflect the influence of dietary protein on the amino acid content of the total serum protein than would the analysis of any pure fraction.

EXPERIMENTAL

The serum protein samples analyzed were obtained in investigations conducted by Melnick, Cowgill and Burack² (9) in which several proteins were assayed for their potencies in the regeneration of serum protein by the plasmapheresis technique. These authors defined the serum protein obtained on the first day of a two week period during which the dog was on a protein-free diet as "normal" serum protein (10) and serum protein secured from bleedings during the second week was considered to be "regenerated" protein from the dog's own tissues. Throughout the third and fourth weeks the diets of the dogs were supplemented by one of the following proteins: casein, lactalbumin, beef serum, and yeast. The third week was allowed for the adjustment to the supplemented protein diet. During the fourth week the various total serum protein samples analyzed were collected.

All of the serum proteins were prepared by precipitation with cold acetone at -5°C . as described by Block (11). The analytical methods used in this investigation were the same as those previously employed (12).

DISCUSSION

The data (Table I) for the various total serum proteins analyzed indicate that there is no appreciable difference between the values for normal serum protein, "reserve" serum protein, and serum protein regenerated by the dogs while on the protein-free diet. The composition of the total serum protein regenerated following the administration of the various individual dietary proteins was the same as that of the normal serum protein. This is of special significance in view of the "turnover"

² We are greatly indebted to these authors who made these proteins available to us for analysis.

in serum protein during the periods of assay. Calculations (9) indicate that by the end of the third week of an assay, about 60 per cent of the circulating serum protein had been newly synthesized as the result of the addition of protein to the diet. By the end of the fourth week, 90 per cent of the circulating serum protein had been newly synthesized.

TABLE I

Analyses of Serum Proteins Regenerated on Various Dietary Regimes

Serum protein sample	Dog	Total nitrogen	Total sulfur	Cystine	Tyrosine	Tryptophan	Histidine	Arginine	Lysine
		No.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Normal	3	12.17		3.21	5.40	2.13	1.8	5.8	9.1
	4	12.12	1.17	3.29	5.53	1.96	1.8	5.7	9.0
Reserve	3	11.55	1.09	3.08	5.68	2.17	1.9	5.8	7.8
	4	11.58	1.20	3.31	6.41	1.41	1.9	5.8	8.9
Regenerated from tissue body protein	3	14.35	1.23	3.13	5.55	1.67	1.7	5.6	7.9
	4		1.17	3.11	5.36	1.70	1.9	5.9	8.2
Following dietary casein	3	12.66	1.11	3.06	5.76	1.82	1.8	6.0	7.8
	4	12.24	1.10	3.13	6.04	1.88			
Following dietary lactalbumin	3	11.92	1.12	3.07	5.92	1.92	1.8	6.0	7.7
	4	12.23	1.15	3.02	5.84	1.87	1.7	5.7	8.2
Following dietary beef serum	3	13.88	1.30	3.21	5.78	1.72	1.9	5.6	8.6
	4	14.10	1.38	3.26	5.70	1.70	2.0	5.5	9.2
Following dietary yeast	3	14.22	1.19	3.11	5.36	1.77	1.9	5.8	8.1

All values except total nitrogen are corrected for ash and moisture as described (12).

Therefore, these authors (9) felt that during the fourth week of collection approximately 75 per cent of the serum protein was newly regenerated protein. The marked increase in serum protein regeneration which occurred following the addition of a single protein to the protein-free diet was taken as evidence that the newly formed protein had its origin from the diet and not from body tissue.

SUMMARY

There was no essential difference in the analyses obtained for the normal serum protein of dogs and that regenerated from various types of dietary protein after plasmapheresis. These results are interpreted as further evidence that dietary protein has little influence upon the composition of the total serum protein.

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The Removal of Inorganic Phosphorus from Phosphate Solutions by Rat Bone

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The presence of a phosphorylating enzyme system in rat and rabbit bones has recently been postulated by Gutman and Gutman (1). These authors added a phosphate-glycogen mixture to the minced ends of the long bones of growing rats and rabbits, and observed a rapid disappearance of inorganic phosphate from the substrate. This loss of inorganic phosphate was attributed to a phosphorylating enzyme system similar to phosphorylase which according to Cori (2) and others mediates the following reaction.



Similar losses of inorganic phosphate under much the same experimental conditions have been observed in this laboratory. The interpretation of the results, however, need not involve the action of a phosphorylating enzyme system.

EXPERIMENTAL

After removing the soft tissue from the humeri, femora, and tibiae of rats 20 to 60 days old, the proximal and distal ends of these bones were finely minced with scissors. Portions of the mixed bone were placed in tared tubes and the weights of the samples determined. In some experiments the bones from as many as three rats were used, although generally sufficient material was obtained from one animal.

A phosphate buffer ($M/45$, pH 7.2) was used as substrate either alone or after the addition of glycogen, phlorizin, NaF or combinations of these. After mixing the minced bone and buffer in about a 1 to 2 ratio and removing a sample, the tubes were stoppered and placed in a water

bath at 37.5°C. Additional samples were taken at various intervals for inorganic phosphate analysis.

TABLE I
Removal of Inorganic Phosphorus from Various Phosphate Substrates
by Minced Rat Bone
pH 7.2. Temp. 37.5°C.

Exp No	Composition of substrate	Time of incubation	Inorganic P, mg.	
			per c c substrate	Loss $\frac{\text{c c. substrate}}{\text{g. bone}}$
1*	P, G	min.		
		0	0.733	
		60	0.580	0.440
1a	P	0	0.733	
		60	0.557	0.460
1b	P, G, F	0	0.733	
		60	0.572	0.460
1c	P, G, Ph	0	0.740	
		60	0.511	0.573
1d	P, G†	0	0.725	
		60	0.572	0.411
2†	P, G	0	0.764	
		60	0.542	0.957
2a	P, G§	0	0.778	
		60	0.611	0.632

P = M/45 phosphate.

G = 0.4% glycogen.

Ph = M/100 phlorizin.

F = M/40 NaF.

* In Exps. 1, 1a, 1b, 1c, and 1d, bone material from three 25 day old rats was used.

† In this experiment bone material from one 40 day old rat was used.

‡ Bone heated in stoppered tube in boiling water bath for 30 minutes before adding substrate.

§ Shafts of the bones only.

These determinations were made on trichloroacetic acid filtrates by the Fiske and Subbarow method (adapted to the Klett-Summerson photo-

electric colorimeter) except that the amidol reducing agent described by Allen (3) was used.

RESULTS

Representative results are given in Table I. The data indicate that the loss of inorganic phosphate from the buffer substrate must be governed by mechanisms other than enzyme action. Heating minced bone in a stoppered tube for 30 minutes in a boiling water bath previous to the addition of the buffer had no inhibitory effect on inorganic phosphate loss. Similarly the presence or absence of added glycogen in the buffer did not alter the loss of phosphate.

The finding of Gutman and Gutman (1) that NaF does not affect the disappearance of phosphate was confirmed. Contrary to their finding, however, there was no indication that $M/100$ phlorizin altered the reaction.

Shaft bones alone showed only slightly less activity than bone ends.

When trichloroacetic acid filtrates of substrates depleted in inorganic phosphate were hydrolyzed 30 or 45 minutes with $N H_2SO_4$, only insignificant increases in phosphate (0 to 4 per cent) were found. This is also contradictory to the findings of Gutman and Gutman who, although they did not identify glucose-1-phosphate, stated that on some occasions over 50 per cent of the phosphate reappeared after 30 minutes hydrolysis.

DISCUSSION

Since the disappearance of inorganic phosphate observed in these experiments cannot be due to enzyme action, a physico-chemical mechanism may be postulated. At least two possibilities suggest themselves: 1. adsorption of sodium phosphate, and 2. an ionic interchange.

West and Judy (4) showed that when tricalcium phosphate is placed in a citrate buffer, the latter ion interchanges to a marked degree with the phosphate ion. The results reported here may be accounted for by assuming an exchange in the opposite direction; *i.e.*, phosphate ion of the buffer for citrate, carbonate, or other ion of the minced bone.

Since the minced bone contained the most proximal and distal extremities of the diaphyses, the entire epiphyses and the intervening cartilage, sufficient bone salts were present to allow such an ion interchange to proceed.

The fact that no significant amount of the removed phosphate could

be recovered by acid hydrolysis points to the absence of glucose-1-phosphate in the substrates.

That the procedure outlined here is not a test for an enzyme system is obvious. It is possible, however, that enzymatic phosphorylation may have proceeded in some of the samples, but the effect was overshadowed by the action of other mechanisms alluded to previously. Since glycogen apparently plays an important part in the process of calcification it would seem logical to ascribe a major rôle to a phosphorylase enzyme system. Gutman, Warrick, and Gutman (5) have recently advanced evidence for its rôle in *in vitro* calcification.

SUMMARY

When a phosphate buffer is added to minced rat bone there is a rapid decrease in the inorganic phosphate content of the buffer. Under the experimental conditions described, this phenomenon cannot be due to enzymatic action. The possibility of an ionic interchange to account for the results is discussed.

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A Dietary Factor, Essential for Guinea Pigs¹

I. Isolation from Raw Cream

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Investigations by Wulzen and Bahrs (1, 2) revealed that guinea pigs developed degenerative changes in skeletal musculature when the animals were raised on grain diets lacking in green feeds, but adequate in other respects including the necessary vitamins. When fresh kale or fresh alfalfa were included in the diet, the deficiency disease did not develop.

Raw or pasteurized skimmed milk, to which had been added 10% of skimmed milk powder, adequate amounts of copper, iron, carotene, orange juice, and straw, and iodized salt *ad. lib.*, also was found to be a deficient diet, as was demonstrated by the development of the typical syndrome (3).

The first sign of the deficiency was the development of stiffness in the wrist. This syndrome increased in severity until it was impossible to bend the wrist. Upon autopsy the muscles were found to be extremely atrophied and in most cases were streaked with closely packed, fine, white lines of calcium deposits running parallel to the muscle fibres. There were often lumps of calcium phosphate deposited under the skin, in the joint regions, between the ribs and indiscriminantly in many body organs, including heart and aorta. Cod liver oil accelerated the onset of the deficiency disease and aggravated the symptoms. In some respects the symptoms suggested those reported for vitamin E deficiency (4, 5, 6, 7). However, vitamin E supplied either in the form of wheat germ oil or as the synthetic alpha-tocopherol² did not cure or prevent the

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deficiency disease. The "grass juice factor" of Kohler, Elvehjem, and Hart (8) was also inactive.

The syndrome could be alleviated within five days by feeding the affected animals 1 g. of raw cream (0.3 g. dry weight) per day. If the cream was heated in the presence of oxygen, the curative properties were destroyed. Cream heated in an atmosphere of nitrogen retained its full activity. Later investigations by Gouley (9) indicated the presence of methyl vinyl ketone in the raw cream as the active curative principle. Synthetic methyl vinyl ketone proved to be curative in a dosage of 5 mg. for six consecutive days, but had at the same time very toxic effects. We could not confirm the presence of methyl vinyl ketone.

Using raw cream as our starting material we were able to isolate in a highly concentrated and probably nearly pure form a factor which in a daily dosage of 0.1 γ was able to cure the stiffness induced by the milk diets within five days.

EXPERIMENTAL

Method of assay. Weaned guinea pigs were raised on a diet of the following composition:

Skimmed milk	100 cc.
Skim milk powder	10 g.
Ferric chloride	0.82 mg.
Copper sulfate	0.78 mg.

Orange juice (1 cc. per 100 g. body weight) and carotene (150 I.U.) were given daily to each animal.

The described syndrome developed in about a month. These animals were used for the test of the activity of the different fractions in the following way:

The foreleg of the guinea pig on the opposite side from the experimenter was extended posteriorly, close to the body wall of the animal, by pressing the thumb on the olecranon process and at the same time supporting the proximal and distal portions of the leg with the fingers. The leg should be as straight as possible. The disengaged hand of the operator was then used to superextend the foot gently by pressing upward on its medial aspect. The foot of a normal animal would bend easily until it formed a right angle with the leg. The nutritionally deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending.

The angle between the foot and the leg varied from 90° in unaffected animals to 180° in very sick animals. This stiffness disappeared if active fractions were administered to the animals.

The active compound is fat soluble. The fractions to be tested were therefore dissolved in Wesson oil, which in itself is inactive, as was proved by preliminary experiments. In order to express activities in a quantitative way, it was necessary to adopt a unit of activity. We have therefore arbitrarily defined one guinea pig unit as follows: a solution of an active fraction in Wesson oil contains one unit per cc. if, when 1 cc. is administered daily for five consecutive days to a sick animal, it cures the affected animal in this time, the stiffness being determined as described above. For each set of determinations three animals were used.

Isolation. a) Churning. Fifteen gallons of raw cream (1 U./g.; total 51,000 U.) were churned and the resulting butter was washed twice with water and pressed free from the wash-liquid.

Yield: 30 kg.; 1.5 U./g.; total 45,000 U.

b) Saponification. The butter was added to a boiling solution of potassium hydroxide in ethanol (20%) and refluxed for four hours in an atmosphere of nitrogen. After cooling, the reaction mixture was acidified with a 5% sulfuric acid solution and the fatty acids separated. These were washed several times with water until free from sulfuric acid.

Yield: 20 kg.; 2 U./g.; total 40,000 U.

c) Steam distillation. The fatty acids were distilled with steam in an atmosphere of nitrogen for twelve hours. The steam distillate was extracted with peroxide-free ether. An aliquot was dried with anhydrous sodium sulfate and used for determining the activity. The remaining solution was immediately used in the next step.

Yield: 175 g.; 200 U./g.; total 35,000 U.

d) Extraction with potassium hydroxide solution. The ether extract was treated with a 5% potassium hydroxide solution till the water layer stayed clear on acidifying. The ether solution was washed free from alkali with water and dried over anhydrous sodium sulfate. The ether was distilled in an atmosphere of nitrogen.

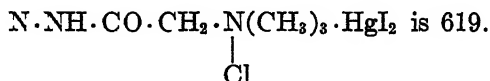
Yield: 66 g. of a yellow oil; activity 500 U./g.; total 33,000 U.

e) Treatment with trimethyl acethydrazide ammonium chloride (10). Sixty-six grams of oil (fraction d) were dissolved in 100 cc. of a 10% glacial acetic acid-absolute ethanol mixture and refluxed with 5 g. trimethyl acethydrazide ammonium chloride for seven hours. The condenser was fitted with a drying tube filled with anhydrous calcium chlo-

ride. Meanwhile the quantity of 1.0 *N* sodium hydroxide solution required to bring 50 cc. of the original acetic acid-ethanol mixture to a pH between 6.5 and 7.0 was determined. This volume of alkali was added to a 500 cc. Erlenmeyer flask, containing 50 g. of ice. At the end of the reflux period the contents of the reaction flask were transferred to the Erlenmeyer flask. This mixture was then continuously extracted with peroxide-free ether for 36 hours. The contents of the extractor were now acidified with 1.0 *N* sulfuric acid solution and again extracted with peroxide-free ether for twenty-four hours. The ether solution was dried over anhydrous potassium carbonate. The ether was distilled under nitrogen.

Yield: 62 mg.; 500,000 U./g.; total 31,000 U.

f) *Precipitation of the mercuric iodide complex of trimethyl acetylhydrazide ammonium chloride.* Sixty-two milligrams of fraction c were treated according to the procedure described by Hughes (11). The crystalline precipitate was filtered, dried and twice recrystallized from 95% ethanol. The melting point of the mercuric iodide complex was 60–61° (uncor., Berle block). The yield was 105 mg. The nitrogen content and the molecular weight were determined. Determinations of the carbon and hydrogen content were not possible because of the presence of mercury in the compound. The molecular weight as determined after the procedure of Rast, with camphor as solvent, was 820. The molecular weight of the side chain



The active principle must therefore contain only one carbonyl group. Its molecular weight is around 200.

N calculated on the above basis	5.12%
N determined	4.88%
Molecular weight calculated	860
Molecular weight determined	820

The mercuric iodide complex of methyl vinyl ketone was prepared. After recrystallization from 95% ethanol, the compound melted at 120–121° (uncor. Berle block). The active principle which we isolated from the raw cream is therefore not identical with methyl vinyl ketone.

g) *Decomposition with hydrogen sulfide.* Ninety-five milligrams of the

mercuric iodide complex were dispersed in a 1.0 *N* sulfuric acid solution and treated with hydrogen sulfide. After complete precipitation of the mercuric sulfide the solution was freed from hydrogen sulfide. The solution was extracted with peroxide-free ether and the ether extract dried over anhydrous sodium sulfate. After removal of the ether *in vacuo* a pale yellow oil resulted, which was active in a 0.1 γ dosage.

Yield: 3 mg.; 10,000,000 U./g.; total 30,000 U.

h) Pasteurized cream. Following the same procedure as outlined in the steps a-e, a crystalline mercuric iodide complex could be isolated. After repeated recrystallization from 95% ethanol the compound melted at 171-172° (uncor.). After decomposition with hydrogen sulfide the resulting oil proved to be inactive.

The authors wish to express their appreciation to Mr. Robert Wong for the nitrogen analyses.

SUMMARY

A procedure for the isolation of a factor present in raw cream, which cures an induced stiffness in guinea pigs has been described. The compound has a high biological activity. This factor is not identical with methyl vinyl ketone, as was previously reported. The compound has a molecular weight of around 200 and contains a carbonyl group.

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The Degradation of Glucose by *Staphylococcus Albus*¹

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Dental caries has been considered of bacterial origin for many years (1, 2). Since it is thought that micro-organisms play a major rôle in the disease, many investigators have attempted to find bacteria that are primarily responsible for the condition. Rodriguez (3) first suggested that *Lactobacillus acidophilus* was the primary cause of the disease. It was later shown by Bunting (4) and co-workers that this was the primary organism associated with dental caries, providing a definite technique is used. By means of another technique Anderson (5) has shown that certain acid streptococci are at least as prevalent as *Lactobacillus acidophilus*.

When one considers the nature of the chemistry involved in the production of acid in the oral cavity (6, 7, 8), and the bacterial flora of the mouth, it is obvious that there are numerous possibilities of symbiotic relationships (9) which would materially diminish the probability of any single organism being responsible for the dental caries.

When it was shown that carbohydrate degradation under the influence of bacterial enzymes followed a more or less definite scheme of reactions (10, 11) similar to carbohydrate degradation under the influence of muscle tissue enzymes (12, 13), it was suggested that the same type of reactions occurred in the mouth for the production of lactic and other acids. The intermediates of these reactions were subsequently isolated from fermenting saliva-glucose mixtures (14).

Since it was shown that these reactions do occur in the mouth, it was thought that if the rates of reaction of each step in the degradation of

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carbohydrate under the influence of enzymes from each type of organism in the oral cavity were known, the combination or combinations of organisms most responsible for acid production with subsequent dental caries would be known.

To date the rates of reaction under the influence of yeast (15) and *L. acidophilus* (16) have been determined. This paper deals with carbohydrate degradation under the influence of *Staphylococcus albus*.

EXPERIMENTAL PROCEDURE

The general method used to determine the rates of various steps in the reaction was to allow a weighed amount of organisms to act for a given period of time on each intermediate in the reaction involved in the degradation of glucose to lactic acid, and then to analyze the substrate quantitatively for the next intermediate. Each step in the procedure was performed at three pH levels—5.4, 6.7, and 8.6. The first and last values were chosen because the work of Kay (17) and others has shown that the optimum pH for phosphatase is either one or the other. The intermediate value, 6.7, was chosen because this represents the pH of normal resting saliva.

The *Staphylococcus albus* used in this investigation was isolated from human saliva, or from tooth scrapings. The organisms were then isolated in pure culture by the usual methods, and grown on a solid 1% dextrose medium. Since large numbers of organisms were required, the usual methods of culturing did not readily lend themselves to the problem at hand. For this reason, a method whereby large amounts of organisms could be grown was employed (15). In all but steps II and III, the organisms were centrifuged from the harvest liquor, dried in a vacuum desiccator, and powdered finely in a mortar and pestle. In this way, the organisms could be weighed on an analytical balance, thus placing the work on a semi-quantitative basis. While the organisms prepared in this way are obviously not in their active form, the enzymes contained in them are still capable of performing their usual reactions.

I. Phosphorylation of Glucose by Staphylococcus albus

The method of forming and isolating hexose diphosphate and hexose monophosphate was to allow a weighed quantity of organisms to act on a known quantity of a buffered glucose solution and then determine the various intermediates by the analysis for the free phosphate, total phosphate, hexose monophosphate, and hexose

diphosphate. The entire procedure was a modification of that of Harden and Henley (18), wherein the modification consisted of the addition of copper sulfate as indicated by Bauer (19), in order to slow down the dismutation of the formed hexose phosphates. Preliminary experiments indicated that the dismutation of the hexose phosphates was too rapid to permit their accumulation, so that it was necessary to conduct the reactions in .001 molar copper sulfate. This in no way interfered with the formation of the hexose phosphates. The phosphorus was determined by the method of Fiske and Subbarow, and the glucose was estimated by the Benedict quantitative procedure. The pH was meas-

TABLE I
Phosphorylation of Glucose
(mml /liter)(24 hours)

pH	5.4		6.7		8.6	
Trial	1	2	1	2	1	2
pH after incubation	5.4	5.4	6.4	6.5	8.1	8.3
Glucose before	443	443	443	443	443	443
Reducing substances after	422	427	447	441	425	421
Free P before	243	243	243	243	243	243
Free P after	205	207	216	212	213	209
P esterified	38	36	27	31	30	34
Hexosediphosphate formed	29.8	28.4	27.0	31.0	30.0	32.2
Hexosemonophosphate formed	8.1	7.6	0	0	0	1.8
Per cent P esterified	15.4	14.9	11.3	12.7	12.2	13.8
Per cent esterified P						
as Diphosphate	79.4	78.5	100	100	100	96.4
as Monophosphate	20.6	21.5	0	0	0	3.6

ured by the Coleman electrometer, and the hexose diphosphate isolated as the barium salt. The results of this experiment are shown in Table I.

II. Aldolase and Isomerase in the Production of Triose Phosphates

The general methods of Utter and Werkman (20) were used in the demonstration of these two enzymes. The cell-free extract was prepared according to an unpublished method used in Werkman's laboratory wherein cells of *Staphylococcus albus* were intimately ground with powdered glass, and the resulting sludge was extracted with water and centrifuged. The clear liquid thus obtained was found to be very active.

One ml. of this enzyme preparation, 1 ml. of glycine buffer of the appropriate pH, and 1 ml. of .1 M sodium fluoride were suspended in a water bath at 37.5°. Two ml. of hexose diphosphate prepared from the calcium salt was added to

start the reaction. Five ml. of 7 per cent trichloroacetic acid was immediately added to the control tubes, containing the same mixture as described, to stop enzyme action. The tubes were incubated for 2 hours. At the end of this time, 5 ml. of 7 per cent trichloroacetic acid, which had been heated to the bath temperature to avoid changing the equilibrium conditions, were added to each of the reaction tubes. After centrifuging, free and total phosphorus were determined. Alkali-labile phosphate was determined as follows: an aliquot of the mixture was added to an equal volume of 2 *N* sodium hydroxide and allowed to stand for 20 minutes. It was quickly neutralized with sulfuric acid, and the free phosphate again determined.

The difference between the free phosphate before and after the alkali treatment yields the combined quantities of glyceryl aldehyde phosphate

TABLE II
Aldolase and Isomerase in the Production of Triose Phosphates
(mml./liter)

pH	5.4		6.7		8.6	
Trial	1	2	1	2	1	2
Initial Hexosediphosphate..	8.25	8.25	8.25	8.25	8.25	8.25
Final Hexosediphosphate. . .	2.43	2.43	2.43	2.43	2.09	2.05
Free P after incubation . . .	3.56	3.56	4.85	4.85	5.17	5.17
Alkali-labile P	2.26	2.26	0.97	0.97	0.97	1.03
Triosephosphate formed . . .	2.26	2.26	0.97	0.97	0.97	1.03
Phosphoglyceraldehyde formed	0.08	0.10	0.07	0.05	0.07	0.10
Dihydroxyacetone phosphate formed	2.18	2.16	0.90	0.92	0.90	0.93
Per cent triosephosphate as dihydroxyacetone phos- phate.....	96.5	95.6	92.7	95.0	92.7	90.2

and dihydroxyacetone phosphate. The phosphate of the hexose phosphate is unaffected by this mild treatment. The iodine oxidation of Utter and Werkman was used to differentiate between the glyceraldehyde phosphate and dihydroxyacetone phosphate. After the iodine oxidation, the free and labile phosphate was again determined. The phosphate of the phosphoglyceric acid is not saponified by the mild treatment. The difference between the phosphate before and after iodine oxidation gives the measure of the glyceraldehyde phosphate. The results of this experiment are found in Table II.

III. The Production of Phosphoglyceric Acid

The general method of Wood, Stone, and Werkman (10) was employed for this determination. Here the phosphoglyceric acid is iso-

lated as a barium salt when the organism is allowed to act on a mixture of glucose diphosphate, sodium pyruvate, and a phosphate buffer in the presence of toluene and sodium fluoride. The hexose phosphate is necessary as a catalyst, and the sodium pyruvate acts as a hydrogen acceptor. The toluene is used as a preservative, while the sodium fluoride in suitable concentrations protects the phosphoglyceric acid from further breakdown.

In each of several 125 cc. Erlenmeyer flasks, 7 g. of *Staphylococcus albus* paste, 7 ml. of 0.67 *M* phosphate buffer of the proper pH, 8 ml. of 20 per cent glucose, 5 ml. of 2.5 per cent sodium hexose diphosphate, and 1.5 ml. of a 0.2 *M* sodium fluoride, 0.1 ml. of toluene, and 8 ml. of sodium pyruvate, were placed, and the mixture incubated at 37° for 24 hours. The flasks were then chilled and stored for sixteen hours in an icebox. The mixtures were centrifuged and the inorganic

TABLE III
The Production of Phosphoglyceric Acid
(24 hours)

pH	Trial	Barium phosphoglycerate	
		mg. /100 ml.	mm./liter
5.4	1	60.2	1.75
	2	75.8	2.21
6.7	1	145.5	4.24
	2	150.2	4.38
8.6	1	130.0	3.78
	2	125.2	3.65

phosphate precipitated from the supernatant liquid by adding ammonia and treating with 20 per cent magnesium acetate. The resulting precipitate was removed by centrifuging, and an excess of 50 per cent barium acetate solution was added to the clear supernatant fluid. The resulting solution was stored in an icebox. At the end of 24 hours, one-ninth volume of ethyl alcohol was added to complete the precipitation. After standing in an icebox an additional 24 hours, the barium phosphoglycerate was filtered off into previously dried porous bottomed crucibles, and dried and weighed. The results obtained are shown in Table III.

IV. The Degradation of Phosphoglyceric Acid

The degradation of phosphoglyceric acid is usually recognized by the appearance of pyruvic acid and phosphoric acid in equivalent quantities. Despite the fact that in many instances the pyruvic acid is quickly attacked and converted into other degradation products, the determination of the liberated phosphate gives a true index of the amount of

phosphoglyceric acid converted. The 3-phosphoglyceric acid employed in this work was synthesized biologically by the method of Ostern and Guthke (21).

In determining the action of *Staphylococcus albus* on phosphoglyceric acid, the general methods of Neuberg and Kobel (22) were employed. Three grams of calcium phosphoglycerate, 7 ml. of 50 per cent acetic acid, and about 20 ml. of water were put into a 50 ml. Erlenmeyer flask, which was then stoppered and vigorously shaken for about an hour. The calcium in the supernatant liquid was precipitated by the addition of an appropriate amount of sodium sulfate. This was centrifuged. The clear liquid was adjusted to the desired pH with sodium hydroxide and diluted to 35 ml. with distilled water.

One gram of dried *Staphylococcus albus* and 1 ml. of toluene were added to 12.5 ml. of the solution of sodium phosphoglycerate prepared as described above. The mixture was sealed in a large test tube and shaken in a water-bath at 37.5° for twenty-four hours. Control tubes were prepared at each pH exactly as above, except that they contained no organisms.

At the end of the incubation period, the tubes were broken open and the contents analyzed. The proteins were precipitated with trichloroacetic acid and removed. The resulting mixture was analyzed for free phosphate, acetaldehyde, and pyruvic acid. Phosphate analyses were made by the method of Fiske and Subbarow. Acetaldehyde and pyruvic acid were determined by the method of Simon and Neuberg (23) as follows: excess 2,4-dinitrophenylhydrazine dissolved in 2 N hydrochloric acid was added to an aliquot of the deproteinized substrate and the mixture allowed to stand in the incubator for twelve hours. The solution was chilled and the precipitated hydrazones filtered off. The hydrazone of pyruvic acid was separated from that of acetaldehyde by extraction with sodium carbonate solution, and the former re-precipitated by the addition of hydrochloric acid. The respective hydrazones, when present, were filtered into weighed porous bottomed crucibles. Since the filtration of the hydrazones was very slow, allowing evaporation of the liquid so that the inorganic salts tended to crystallize out, it was necessary to recrystallize the hydrazones before weighing. It might be pointed out that other carbonyl compounds in addition to acetaldehyde might also be present in the neutral precipitate. The results obtained at the various pH values are given in Table IV.

V. Conversion of Pyruvic Acid to Lactic Acid

The general method used to determine the reduction of pyruvic acid was to permit the organisms to react with a sodium pyruvate substrate in a suitable buffer solution containing a hydrogen donor. After a suitable incubation time the reaction mixture was analyzed for pyruvic acid, lactic acid, and acetaldehyde.

Preliminary experiments indicated that the pyruvic acid was destroyed very rapidly, so it was necessary to decrease the incubation time

to one hour in order to prevent the pyruvic acid from being completely destroyed.

Ten ml. of buffer solution, one ml. of 0.002 molar sodium pyruvate, and one ml. of *Staphylococcus albus* paste was well mixed and allowed to incubate for one hour at 37.5°C. At this time the protein was removed and the cell-free filtrate was analyzed for lactic acid by the method of Miller and Muntz (24) as modified by Barker and Summerson (25). Pyruvic acid and acetaldehyde were determined by the method of Clift and Cook (26). Insofar as work on other organisms has indicated that a hydrogen donor is necessary with some organisms (15), calcium hexose diphosphate was added to some of the substrates. It was found that although about 80 per cent of the pyruvic acid was destroyed in one hour in a phosphate buffer, no lactic acid was present at this time.

TABLE IV
The Degradation of Phosphoglyceric Acid
(mmol./liter) (24 hours)

pH	Trial	H ₃ PO ₄	CH ₃ ·CO·CO·OH	CH ₃ ·CHO*
5.4	1	8.10	None	1.67
	2	8.20	None	1.82
6.7	1	7.28	None	0.86
	2	7.16	None	1.34
8.6	1	5.37	None	0.50
	2	6.15	None	0.59

* This includes all non-acidic carbonyl compounds, calculated as acetaldehyde.

The experiment was repeated, using sterile human saliva as the buffer solution, with results similar to that found in a phosphate buffer. The results are shown in Table V.

In 1937, Krebs (27) showed that *Staphylococcus albus* is among the bacteria which do not form lactic acid from pyruvic acid by simple reduction in the presence of a hydrogen donor. He showed, rather, that two moles of pyruvic acid are converted to one mole of lactic acid, one mole of acetic acid, and one mole of carbon dioxide. He further showed that the lactic acid formed is oxidized to acetic acid in the same substrate under aerobic conditions. For this reason, the reaction was performed in an apparatus especially designed to maintain anaerobic conditions. The reaction was carried out in both the phosphate and the bicarbonate buffers. In this case, although very little pyruvic acid was decomposed,

lactic acid was formed almost in a ratio of one mole of lactic acid to one mole of pyruvic acid. In the bicarbonate buffer, lactic acid was formed in the ratio of one mole of lactic acid to two moles of pyruvic acid. The results are shown in Table VI.

TABLE V
The Conversion of Pyruvic Acid to Lactic Acid (Aerobic)
(mm./liter)

Buffer	HDP ^a Added	Pyruvic acid		Lost	Lactic acid Found	CH ₃ ·CHO Formed
		Original	Final			
Phos. pH 6.7	None	5.84	1.40	4.44	None	1.0
	None	5.90	1.30	4.60	None	0.9
	6.00	5.94	1.70	4.24	None	1.1
	6.00	5.94	1.72	4.22	None	1.0
Bicarb. pH 6.8	None	6.01	5.07	0.94	None	0.5
	None	6.05	5.53	0.92	None	0.5
	6.00	6.20	5.04	1.20	None	0.4
	6.00	6.20	5.12	1.08	None	0.4
Saliva (ster- ile)	None	6.44	2.22	4.22	None	1.4
	None	6.44	2.26	4.18	None	1.2

* Hexosediphosphate.

TABLE VI
The Conversion of Pyruvic Acid to Lactic Acid (Anaerobic)
(mm./liter)

Buffer	Original	Pyruvic Acid		Lactic Acid Formed	CH ₃ ·CHO Formed
		Final	Lost		
Phosphate pH 6.7	2.02	1.88	0.14	0.17	0.08
	2.02	1.80	0.22	0.20	0.01
Bicarbonate pH 7.0	2.49	1.61	0.88	0.45	None
	2.49	1.58	0.91	0.48	None

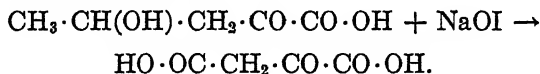
VI. The Fate of Pyruvic Acid

Insofar as the degradation of pyruvic acid was much different under the influence of the enzymes of *Staphylococcus albus* than with any other organisms studied in this laboratory, it was thought interesting to isolate and identify the products formed. Furthermore, it was noticed that the reaction mixture had a particularly foul odor, which was not characteristic of any known product of fermentation.

Larger runs of the reaction were made, wherein only phosphate buffer pH 6.8, organisms, and sodium pyruvate were present. The reaction was permitted to go to completion, and the reaction mixture was tested by various means. It was found that ether extraction would remove the acetaldehyde, pyruvic acid, and perhaps traces of lactic acid, leaving a solution which contained two organic components. One of the components was acid and the other was neutral. Neither could be isolated in pure form without decomposition.

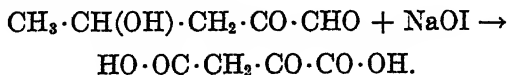
The Acid Fraction

The acid fraction was isolated as the sodium salt, which decomposed upon heating. The neutral equivalent of the free acid was 130. It formed a 2,4-dinitrophenylhydrazone, M.P. 218–225°C., corrected, and contained 18.12 per cent nitrogen. When treated with sodium hypoiodite it formed a dibasic acid, M.P. 149–152°C., which in turn formed a 2,4-dinitrophenylhydrazone, M.P. 204–209°C., corrected. A nitrogen analysis on the hydrazone gave 17.80 per cent nitrogen. The product of the iodoform reaction corresponds with oxaloacetic acid, which according to Clift and Cook (28) forms a 2,4-dinitrophenylhydrazone, M.P. 205–211°C., and the calculated value for nitrogen is 17.94 per cent. On the basis of the above, the acidic compound formed is a α -keto- γ -hydroxyvaleric acid. This compound should form a 2,4-dinitrophenylhydrazone with nitrogen content of 17.94 per cent. The formation of oxaloacetic acid could be represented as follows:



The Non-acidic Compound

The non-acidic compound was never isolated in pure form, as it was exceedingly unstable, but formed a white crystalline dioxime which melted at 203–205°C. The dioxime contained 17.82 per cent nitrogen. When treated with sodium hypoiodite, a solution containing the compound also formed oxaloacetic acid which was identified as the 2,4-dinitrophenylhydrazone. On the basis of this evidence, it is probable that the compound is α -keto- γ -hydroxyvaleraldehyde, and would react with sodium hypoiodite as follows:



This compound would form a dioxime with calculated nitrogen content of 19.1 per cent.

DISCUSSION

The above data indicate that the enzymes present in *Staphylococcus albus* are capable of promoting the reactions set forth in the Embden-Meyerhof scheme of carbohydrate degradation, although many side reactions take place following the pyruvic acid stage. It is quite evident that there is a wide variation in the rates of the various reactions involved. The amount of phosphorylated hexose is quite small when compared to that produced by yeast under the same conditions, but is in about the same order as that produced under the influence of *Lactobacillus acidophilus*.

The conversion of phosphoglyceric acid to pyruvic acid proceeds smoothly, but is probably much slower than the destruction of pyruvic acid, as in no case was pyruvic acid detected in the reaction mixture. There is no reason to suspect that pyruvic acid was not formed, as the next phase of the reaction demonstrated that pyruvic acid was rapidly destroyed.

At this point in the chain, an interesting reaction occurred. Instead of forming lactic acid under aerobic conditions, there was apparently an aldol condensation between the acetaldehyde and pyruvic acid to form α -keto- γ -hydroxyvaleraldehyde. This compound is of much interest, as it has been taken for granted as an intermediate in the production of fats from carbohydrates. This mechanism was first suggested in 1913 (29), but the aldehyde had not been isolated or identified. Insofar as these compounds were formed only in phosphate buffer and probably in saliva mixtures, it is quite probable that the phosphate ion may play a major rôle in the reaction.

On the basis of the above, it is evident that *Staphylococcus albus* plays no major rôle in dental caries, and on the basis that pyruvic acid is rapidly destroyed, it is probable that it may have a pronounced inhibiting effect.

SUMMARY

The degradation of glucose under the influence of the enzymes in *Staphylococcus albus* has been investigated. It was found that these enzymes can promote all of the reactions of the Embden-Meyerhof scheme. Lactic acid, however, is produced only under anaerobic con-

ditions. Under aerobic conditions no lactic acid is formed. Two new compounds, α -keto- γ -hydroxy valeric acid and α -keto- γ -hydroxyvaleraldehyde, were found. The former was isolated and identified, while the latter was isolated and identified only in the form of the dioxime. The former compound may be of importance in the conversion of carbohydrate to fat.

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Enzymatic Reduction of Fural and Furoin

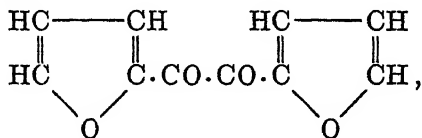
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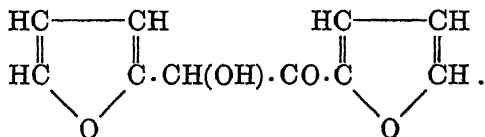
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Neuberg and Nord (1) found that diketones are subject to phytochemical reduction. Diacetyl, $\text{CH}_3 \cdot \text{CO} \cdot \text{CO} \cdot \text{CH}_3$, is hydrogenated by fermenting yeast to *l*-2,3-butylene glycol, $\text{CH}_3 \cdot \text{CH}(\text{OH}) \cdot \text{CH}(\text{OH}) \cdot \text{CH}_3$. Acetyl methyl carbinol (acetoin), $\text{CH}_3 \cdot \text{CH}(\text{OH}) \cdot \text{CO} \cdot \text{CH}_3$, which is readily oxidized in the presence of air to diacetyl, $\text{CH}_3 \cdot \text{CO} \cdot \text{CO} \cdot \text{CH}_3$, yields the same glycol (2). Nagelschmidt (3) demonstrated that the hydrogenation of the diketone is carried out in two steps, and that acetyl methyl carbinol is formed intermediately. Neuberg and Nord further demonstrated, using benzil, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{CO} \cdot \text{C}_6\text{H}_5$, that phytochemical reduction takes place with aromatic diketones (1).

Since this process is of great interest, we have investigated whether it is applicable to the group of *heterocyclics*.¹ We chose for this purpose two representatives of the furfural group, because of their relation to the carbohydrates, *fural*,



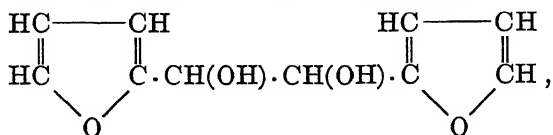
and *furoin*,



¹ From *hydroaromatic diketones* we have tested the 2,3-diketocamphane. In unpublished experiments (with Elisabeth Peiser) it has been established, that the *d*- and the *l*-camphorquinone are transformed to 3-oxycamphor by phytochemical reduction, which proceeds asymmetrically.

Both substances are easily prepared following the method (4) of E. Fischer proceeding from furfural.

The phytochemical reduction leads to *hydrofuroin*,

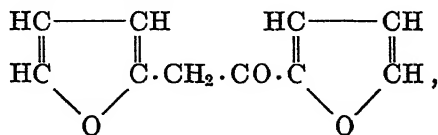


and takes place in a strictly enzymatic manner by means of yeast extract, prepared from Anheuser-Busch or National Grain yeast following the directions recommended by us (5). It proceeds, for most of these biochemical reductions, at least partly asymmetrically.

Starting from furil, furoin is formed first. This reaction presents the most simple as well as the quickest *demonstration of the phytochemical reduction* for lecture purposes. The presence of furoin can be proved, even in great dilution, by its characteristic reaction in aqueous or alcoholic solutions of alkalis (deep blue green color with deep violet red dichroitic iridescence).² This reaction may be carried out in unfiltered fermentation mixtures. It is positive after 30 seconds.³

In the course of our investigation of the phytochemical reduction of polyketones (6), Vercellone examined the behavior of anthradiquinone. This yellow compound which is not readily available, is converted to the red quinizarine. However, the demonstration of the biological process based on this effect is not readily accomplished since the labile tetraketone in aqueous solution is spontaneously transformed to quinizarine by simultaneous oxidation (ring cleavage) of another molecule of the tetraketone to phthalic acid. Furoin is absolutely resistant and, in addition, is commercially available (Eastman Kodak) so that this experiment may be carried out without difficulty.

Dihydrofuroin has not been obtained up to the present by a strictly chemical process because *desoxyfuroin*,



² It is not impossible that this striking color effect is connected with the ketyl reaction described by Schlenk, W., and Thal, A., *Ber. chem. Ges.* **46**, 2851 (1913).

³ This test probably may be used for the demonstration of biochemical reduction processes in other cases.

is formed instead in the course of the chemical reduction. However, electrolytic reduction of furil, analogous to the formation of pinacol from acetone, gives the glycol, probably a mixture of the *d,l*- and the mesoform, as shown by Albert and Lowy (7). It is not mentioned by these authors whether formation of furoin occurs. Despite the analogy between furil and benzil, or furoin and benzoin, biochemically there is a difference, *i.e.*, that benzoin can be submitted to phytochemical reduction (Neuberg and Nord) (1) only with difficulty, whereas phenyl acetyl carbinol is reduced more easily (8). It is a compound which has a position between aliphatic and aromatic acyloines.

PHYTOCHEMICAL REDUCTION OF FURIL

To 100 g saccharose in 1000 cc. of water and 100 g. baker's yeast (Fleischmann), after 5 minutes of fermentation, 3 g. furil in 40 cc. warm alcohol or 20 cc. dioxane are added. (Furil and also furoin do not disturb the fermentation. It seems that both substances have an accelerating effect as do other diketones and carbonyl compounds.) An unfiltered sample gives, after 30 seconds, the characteristic test for furoin. Before introduction of the alcoholic furil solution neither the latter nor the fermentation mixture gave the test for furoin. Further control experiments show that furil is not changed to furoin by a mixture of invert sugar and yeast which was boiled beforehand.

The fermentation mixture is permitted to stand until CO_2 is no longer given off. This is after the elapse of about 24 hours at room temperature. Then 50 g. sugar in 300 cc. water and 10 g. fresh yeast are added. Further fermentation occurs for 24 hours. This is done in order to insure as complete a reduction as possible of furil to furoin, although part of the furoin which is formed, is reduced to hydrofuroin.

The furoin is extracted from the mixture with chloroform (10 cc. of CHCl_3 for every 100 cc.). It is best to extract without filtering since the yeast occludes much furoin. The chloroform layer is separated from the yeast by means of the centrifuge. The centrifuged chloroform solution is distilled off, and a solid is obtained. Since furil is less soluble in alcohol than furoin, this solvent is used for the separation. The alcoholic solution of crude furoin is filtered from the furil and evaporated to dryness *in vacuo*. After recrystallization from toluene and ethanol the m.p. is 130–131° (uncor.). (Synthetic *d,l*-furoin melts at 135°.) The yield amounts to 0.3 g.

The color test with a trace of the substance is distinct. Fehling's and Ost's solution as well as ammoniacal silver nitrate solution are quickly reduced at room temperature. Cupric acetate solution is reduced more slowly.

$\text{C}_{10}\text{H}_8\text{O}_4$. Calculated. C 62.5, H 4.2.

Found. C 62.3, H 4.4.

$[\alpha]_D^{25} = -4.94^\circ$

($\alpha = -1.19^\circ$; $l = 2$; $c = 12.05$; dioxane solution).

The alcohol insoluble portion (1.7 g.) has a melting point of 164°. It is unchanged furil.

The residual mixture, exhausted with chloroform and filtered, upon applying the method of extraction of hydrofuroin to it, yields 0.6 g. hydrofuroin. Thus are recovered from 3 g. furil, 1.7 g. furil, 0.3 g. furoin, 0.6 g. hydrofuroin.

PHYTOCHEMICAL REDUCTION OF d,l-FUROIN

It seems that the antipodes of furoin are reduced at different rates. At least more time and repeated addition of sugar and yeast are required to complete the procedure. The time can be reduced by constant stirring.

To 100 g. saccharose, 1000 cc. water, 100 g. yeast (Anheuser-Busch, Fleischmann, or National Grain), after fermentation starts at room temperature, add 10 g. furoin in 25-30 cc. dioxane. After 24, 48, and 72 hours, 60 g. saccharose in 600 cc. of water and 60 g. fresh yeast are added, and after 4 days 100 g. sugar in 1000 cc. of water. The color test for furoin is negative on the following day, and also negative the test with Fehling's solution. After filtration the mixture is evaporated over a steam bath to 250 cc. The solution is made alkaline with Na_2CO_3 (to retain the succinic acid formed by the fermentation) and saturated with Na_2SO_4 . Then it is extracted five times with 25 cc. of ether. Upon filtration and evaporation of the ether solution a yellow syrup results which is soluble in water, ethanol, benzene, and ether, and insoluble in petroleum ether. After standing for 6 weeks the syrup crystallized. The resulting substance is recrystallized from a mixture of benzene and petroleum ether and yields long, lustrous, white needles of dihydrofuroin; m.p. 60-61° (uncor.). Yield 2.2 g.

$$[\alpha]_D^{25} = -3.64^\circ$$

$$(\alpha = -0.54^\circ; l = 2; c = 7.42; \text{alcoholic solution}).$$

Dibenzoate. 1.4 g. of the syrupy substance are dissolved in 6 cc. of anhydrous pyridine to which 2.2 g. of benzoyl chloride are added. The mixture is heated over a low flame for several minutes. After cooling, 35 cc. water are added. A brown precipitate results. It is filtered off and washed with dilute Na_2CO_3 solution, and then with several portions of water. The substance is dissolved in hot 95 per cent alcohol, filtered, and permitted to stand. A dibenzoate separates. It is filtered and recrystallized with charcoal from 95 per cent alcohol. White crystals, m.p. 180-181°.

$\text{C}_{21}\text{H}_{19}\text{O}_6$.	Calculated.	C 71.65, H 4.5.
	Found.	C 71.7, H 4.7.

(A lower melting dibenzoate precipitated from the filtrate by the addition of water. It is probably another stereochemical form of the derivative.)

THE PHYTOCHEMICAL REDUCTION OF FURIL AS A LECTURE DEMONSTRATION

2 g. saccharose are dissolved in 20 cc. water and 2-3 g. pressed baker's yeast are added. When fermentation has become marked, introduce a few crystals of pure furil (0.05 g.) in 1 cc. alcohol. After 30 seconds (at

least) pour out 2 cc. of the unfiltered mixture and add several drops of aqueous or alcoholic NaOH (40 per cent). After a few seconds a deep green-blue color appears in reflected light and a red violet color in transmitted light. This dichroitic color disappears by shaking with air and reappears when permitted to stand until all furoin is oxidized.

Our thanks are due to the Quaker Oats Company for providing the furfural, to the different yeast companies, and to Prof. J. B. Niederl for carrying out the microanalyses.

SUMMARY

By action of fermenting yeast furoin is reduced to hydrofuroin. Furil acts likewise, forming furoin intermediately. The products of these phytochemical reductions show optical activity.

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The Hypertensive Effect of Diets High in Tyrosine

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There has long been evidence available in the literature that tyrosine might be involved in the etiology of certain types of hypertension. The so-called "blasser Hochdruck" of Volhard (1) was attributed by him to excessive tyramine formation from tyrosine. Subsequently, Heinsen and Wolf (2) found that extracts of blood from patients with nephritis, nephrosclerosis, and contracted kidneys were vasopressor when injected into animals. The extracts gave positive tests which are characteristic for tyramine.

Heinsen (3) and Holtz (4, 5, 6, 7) studied the formation of pressor and depressor amines in various tissues of the body. Recently, Bing and his coworkers (8, 9, 10) have revived interest in the possible interrelation of pressor amine formation from amino acids and hypertension. Production of hydroxytyramine by decarboxylation of dihydroxyphenylalanine was demonstrated.

With these experiments in view, it seemed probable that the blood pressure of normal animals might be affected by diets containing an amino acid composition which would yield on decarboxylation predominantly pressor amines. The logical amino acid to try was tyrosine because of its availability and its decarboxylation to tyramine.

EXPERIMENTAL

Diets were prepared containing 5 and 10 per cent tyrosine. The basal diet was changed with the experiment. Tyrosine used was *l*-tyrosine (Merck and Co.) and a crude preparation obtained from hydrolyzed casein.¹ It was found that no differences existed between rats

¹ Obtained through the courtesy of Dr. Arnold H. Johnson, Research Laboratories, Sealtest, Inc.

on the various diets containing either the crude or the purified *l*-tyrosine; therefore no distinction will be made in the text. To conserve on food consumption, and therefore on the amount of tyrosine needed, young rats weighing 50–60 grams were placed on the various diets. In some instances, the symptomatology was followed until death. In others, the rats were sacrificed at various stages in the syndrome for the determination of blood pressure. The blood pressure was determined under nembutal anesthesia (0.003 cc. of 1 per cent solution per gram of rat). 30 minutes' time elapsed between injection of the nembutal and the time pressure was taken. On rats of this size, the abdominal aorta is the only blood vessel large enough to permit the insertion of a 23 gauge needle. The blood pressure was recorded using an optical manometer and 5 per cent sodium citrate as an anticoagulant. To test the accuracy of the method, a series of 50 determinations were made on normal rats, weighing 50–60 grams. Of these fifty rats, seven were killed through failure to insert the needle properly into the aorta, resulting in puncture and bleeding. The average systolic pressure was 105 mm. within the limits of 80 to 124 mm. (81 per cent within the range of 100 to 120 mm.). To check further the accuracy of the method, 20 rats of the same size were injected with 0.2 cc. of 1:1000 adrenaline, and the blood pressures averaged 119 mm. within the limits of 100 and 145 mm. When injected with 0.4 cc. of this 1:1000 adrenaline solution, the average pressure was 146 mm. within the limits of 113 and 170 mm. It is our belief that the method is accurate to within 5 per cent.

1. Rats on a synthetic diet of vitamin free casein, 18.0 per cent; sucrose, 67.0 per cent; salts, 4.0 per cent; butter fat, 9.0 per cent; and cod liver oil, 2.0 per cent were given supplements of B complex factors added to the basic composition at the following levels: thiamine hydrochloride, 5.0 mg.; riboflavin, 10.0 mg.; pyridoxin, 5.0 mg.; nicotinic acid, 100.0 mg.; calcium pantothenate, 100.0 mg.; choline chloride, 200.0 mg.; inositol, 200.0 mg.; and *p*-aminobenzoic acid, 100.0 mg. 5 and 10 per cent tyrosine was added to this basic composition replacing an equivalent amount of sucrose. At the 10 per cent tyrosine levels, a syndrome develops which runs a course of approximately two weeks. Symptoms are manifested in the second week. The first indication is in a blanching of the paws, which appear bloodless a condition which it is felt corresponds to the "blasser Hochdruck" (1). Then the paws become edematous and reddened; the edema extends to the head and shoulders. The animals assume a hunched posture and walk painfully.

The eyes become involved; an exudative blepharitis is seen in which the exudate is dark brown in color, suggesting melanin.

At the 5 per cent level the same symptomatology is seen, but the animals survive for three or four weeks, the onset of the syndrome being delayed.

Blood pressure determinations were made on these animals at all stages in the syndrome. With few exceptions, it was found that high blood pressure is in evidence at the stage of the blanching of the feet and diminishes thereafter so that determinations on animals showing the marked edema were more frequently within a normal range. During the phase of blanching of feet, the blood pressure averaged 147 mm. for a total of 30 rats, within the range of 135 to 180 mm. During the phase of marked symptoms, the blood pressure averaged 114 mm. for 58 rats, within the range of 100 to 145 mm. It is felt that the 114 mm. pressures averaged for the rats during the phase of severe symptoms are above normal and definitely so in those cases exceeding the maximum value obtained for the normals, namely 124 mm. During the phase of "blasser Hochdruck," the hypertensive effect of the tyrosine in the diet is marked, being increased by 30 per cent.

2. When the tyrosine was added at 10 per cent to the McCollum stock diet the results seen were the same, excepting an apparent increase in the intensity of the syndrome. This suggested a dietary factor associated with acute toxicity manifestations, and in order to check this several other diets were used.

3. Diet of composition the same as in series 1 but with 2 per cent ascorbic acid added. The ascorbic acid seemed to have no effect on the severity of the symptoms. In five rats, 50 mg. of ascorbic acid was injected daily in addition to that received by the rat in the diet. These animals showed no alleviation of the severity of the syndrome.

4. A diet with the composition of the series 1 set, but with 5 per cent yeast replacing 5 per cent of the sucrose, was fed to 20 rats. Here, again, the severity of the syndrome was not lessened. It would seem that neither ascorbic acid nor the B complex in the form of yeast altered the toxicity of the tyrosine in any way, under the conditions of this study. In all instances where the diet was modified, the blood pressures were elevated if taken during the proper phase of the syndrome.

DISCUSSION

Sullivan, Hess, and Sebrell (11) and Lillie (12) reported that diets containing 2.5 to 20 per cent tyrosine resulted in a syndrome charac-

terized by reddening and edema of the extremities, exudative blepharitis, hepatic fatty changes and minor grades of parenchymatous degeneration of the kidney. The eye involvement was described as a breaking down of the center of the crystalline lens to amorphous oxyphil debris with lymphocyte infiltration of the iris and ciliary body, and slight polymorphonuclear infiltration of the cornea. The work is in some measure complicated by the use of a basic diet containing 4 per cent casein, which resulted in a moderate centrolobular fatty degeneration in the liver. These observations as to the symptomatology are entirely confirmed by our work, and it is suggested that the hypertension associated with the high intake of tyrosine is responsible for the symptomatology and histo-pathology seen. In one or two instances, cerebral hemorrhages were noted in these rats on gross inspection. The edema, frequently reaching a state of anasarca, can best be explained by elevation of capillary blood pressure exceeding that of the osmotic pressure of plasma colloids with resultant forcing of water into the tissues. It is possible that the albuminuric retinitis of human hypertensive states may be associated with the exudative blepharitis seen in the rat.

A decarboxylase for tyrosine (4) was demonstrated to exist in the kidney, but not in the liver or pancreas of various animals. It is tentatively suggested that the tyrosine decarboxylase of the kidney forms a concentration of tyramine locally, thus precipitating a localized ischemia similar to that produced by constriction of the renal arteries (13), which accentuates the hypertensive effect. This suggests the principle that where the greatest concentration of the decarboxylase is, there will be the greatest effect of the potentially hyper- or hypotensive agent.

Toxicity has been demonstrated for glycine (14), cystine (15), lysine (11), tryptophan (11), tyrosine (11), serine (16), and methionine (17) given at varying levels. Serine (16) causes anorexia, albuminuria, redness of the feet, hemorrhages under the nails, weight loss, and death. It was suggested that peripheral circulatory failure with marked congestion of liver and lungs and severe damage to the renal tubules resulted. There is a certain similarity between the syndrome of serine and tyrosine poisonings. At present, no claims of specificity in the production of the syndrome are made.

SUMMARY

l-Tyrosine as a dietary component in concentrations of 5 and 10 per cent produces a syndrome in rats characterized by hypertension.

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The Chemistry of Infectious Diseases: VI. Changes in the Blood Serum Proteins of Dogs During Type I Pneumococcal Pneumonia

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Investigations of the blood serum proteins in human pneumonia have shown that a decrease in blood protein concentration is common (1, 2); that, at times, an inversion of the albumin-globulin ratio occurs (2), and that albuminuria frequently accompanies the febrile stages of the disease (3, 4). It is the purpose of this investigation to ascertain whether these changes in blood protein concentration are also encountered in experimental pneumonia in dogs, and if so, what relationship exists between the time of inoculation of the pneumococci and the occurrence of the abnormal blood values. In addition, such a study affords an opportunity to ascertain if the previously observed loss in cystine content of hydrolyzed dog sera during pneumonia (5) is entirely due to a corresponding decrease in total serum proteins, or whether actually a change in the cystine composition of these proteins occurred.

EXPERIMENTAL

The dogs used in this investigation were mongrels weighing from 18 to 35 lbs. They had been wormed at least three weeks prior to the start of the experiment and their blood serum gave an entirely normal polarographic protein wave (6, 7). Prior to infection, enough blood was drawn daily from the external jugular veins to give 5 cc. of serum. After a normal level in serum values was established, the dogs were infected with Type I pneumococci as previously described (6).¹ Throughout the

¹ The inoculation of the dogs with the pneumococci was again performed by Dr. W. Harry Feinstone of this laboratory, to whom we wish to express our gratitude.

course of infection daily roentgenograms of the lungs were taken to ascertain the degree of consolidation and eventual resolution. Daily blood samples were drawn until the animal either had recovered, or had died.

The serum proteins were fractionated according to Kingsley's method (8) modified in order to make simultaneous determinations of cystine and nitrogen possible. These modifications, while extensive in some phases, introduced no error, as evidenced by nitrogen analyses of the same serum sample prepared by both the original and the modified procedures.

Total Serum Proteins

5 cc. of freshly prepared 20 per cent trichloroacetic acid was added to a 15 cc. conical, ground-glass jointed centrifuge tube² which contained 0.5 cc. of serum, diluted with 2.5 cc. of water. After the solution was mixed and allowed to stand for at least 15 minutes, it was centrifuged at 2500–3000 r.p.m. for 15 minutes and the particle-free supernatant was decanted. 2.5 cc. of an HCOOH-HCl digestion mixture (9) was added and the solution refluxed at 118–125° for 18 to 22 hours. The cooled hydrolyzate was then transferred quantitatively to a 10 cc. volumetric flask and made to volume with 5 *N* HCl. Triplicate cystine determinations were run on 1 cc. aliquots by Vassel's (10) method while the remainder of the solution was used for duplicate micro-Kjeldahl nitrogen analyses.

Albumin-Globulin Fractionation

1.3 cc. of serum, 19.5 cc. of 23 per cent Na₂SO₄ solution at room temperature and 10 cc. of U.S.P. ether (8) were added to a 50 cc. centrifuge tube fitted with a ground-glass joint. The tube was stoppered, shaken vigorously for 0.5 minutes, and, after standing for 15 minutes, centrifuged at 2500–3000 r.p.m. for 15 minutes. The globulins floated as a solid cake between the aqueous salt and ether layers. A pipette, drawn out at least 10 cm. to a fine bore, was introduced into the lower liquid past the globulin cake. This was best accomplished by tilting the centrifuge tube until the globulin cake floated freely without touching one side of the glass tube. 13 to 15 cc. of the lower liquid was withdrawn, care being exercised not to break the globulin cake. The al-

² These centrifuge tubes were made to specifications by Ace Glass Inc., Vineland, N. J.

bumins from 10 cc. of the withdrawn liquid, after dilution with 20 cc. of water, were precipitated in another 50 cc. ground-glass jointed centrifuge tube with 5 cc. of 10 per cent trichloroacetic acid. After being vigorously shaken, the mixture was allowed to stand for 30 minutes and was then centrifuged at 2500-3000 r.p.m. for 15 minutes. If particles floated on the surface of the liquid, the mixture was reshaken and re-centrifuged. Rarely was it necessary to repeat this treatment a third time. The particle-free supernatant was carefully decanted and the precipitated proteins hydrolyzed with 2.5 cc. of the HCOOH-HCl digestion mixture. The hydrolyzate was treated in the same way as described for total proteins. Values thus obtained were for the albumin fraction, while the globulin values were calculated as the difference between those of the total proteins and the albumins.

RESULTS AND DISCUSSION

Two of the six dogs used did not develop typical symptoms of pneumonia; consequently they have been omitted in the consideration of the data, except where otherwise noted. The remaining four dogs developed pneumonia of varying degrees of severity which was fatal in two cases. Death in one of the latter was, however, not primarily due to the infection. X-ray films of the previously consolidated areas of the lungs showed almost complete resolution prior to death and all other clinical symptoms pointed towards an uneventful recovery, when jaundice developed and the animal died soon thereafter.

In accordance with previous observations (6), a sudden and sharp rise in temperature occurred in every case between the 12th and 18th hour after infection. During this period and during the following 24 hours, the concentration of the serum proteins remained normal. Independent of the consequent severity of the infection, the sera of all dogs showed the first change from normal at about the 50th hour after the inoculation of the pneumococci. Thereafter the serum values became progressively abnormal until, soon after the crisis, a trend towards normal serum protein concentrations began. This occurred between the 90th and 160th hour after infection. It would appear that the final, most abnormal levels in serum protein concentrations were approached most rapidly by the most severely infected dogs and that their period of recovery also was the longest. This is in accordance with previous findings where the return of the polarographic serum protein wave height to normal values was compared to the severity of the infection (6). One

of the two dogs which survived the infection required only 250 hours for complete recovery, while the second animal needed over 700 hours to attain normal serum protein concentrations again.

During the course of the infection the albumin concentration fell to about one half of its normal range, whether the values were calculated from nitrogen or from cystine determinations. During this same period the globulin content of the serum increased at the height of the infection to a maximum of roughly twice its normal value, and here again values obtained from cystine analyses followed the same trends as those from nitrogen determinations. In Fig. 1, Curves I-IV, the variations in serum albumin and globulin concentration ($\text{g. N}_2 \times 6.25$ per 100 cc. of serum) throughout the course of the infection were plotted against hours after infection for the four dogs. In each of these, as well as in subsequent curves, the limits of normal variability encountered during a 2 to 3 week period previous to infection were plotted as broken, parallel lines; those with small dashes representing the highest and those with large dashes the lowest normal values. It is quite evident from an inspection of Curves I to IV in Fig. 1 that a pronounced fall in albumin concentration and a simultaneous increase in globulin content began in all four dogs at about the 50th hour after infection. Similar graphs were obtained when mg. of albumin-cystine or globulin-cystine per 100-cc. of serum were plotted against time after infection instead of g. of protein. This is demonstrated for Dog 31 as a representative example in Fig. 1, Curve V. This graph should be compared with that shown in Curve IV in Fig. 1, the latter representing the albumin and globulin concentrations of the same dog but calculated from nitrogen values.

The albumin-globulin ratios of the sera of the four dogs throughout the course of the infection are shown in Fig. 2, Curves I to V. The ratios in Curves I to IV were calculated from the nitrogen values in the customary manner, while those in Curve V were obtained by dividing mg. of albumin cystine by mg. of globulin cystine, each per 100-cc. of serum. As a typical example, the data obtained in this manner from the sera of Dog 31 are shown in Curve V. This graph should be compared with Curve IV of Fig. 2, the latter representing the corresponding ratios of the sera of the same dog but determined from nitrogen analyses. It is evident from these graphs that, within the limits of experimental error, the curves tend to fall and rise similarly, whether nitrogen or cystine values are used; it is equally evident from the magnitude of these changes during infection that separate determinations of the albumins and

globulins as criteria of changes in protein concentration give a truer picture than the mere estimation of total serum proteins that are often

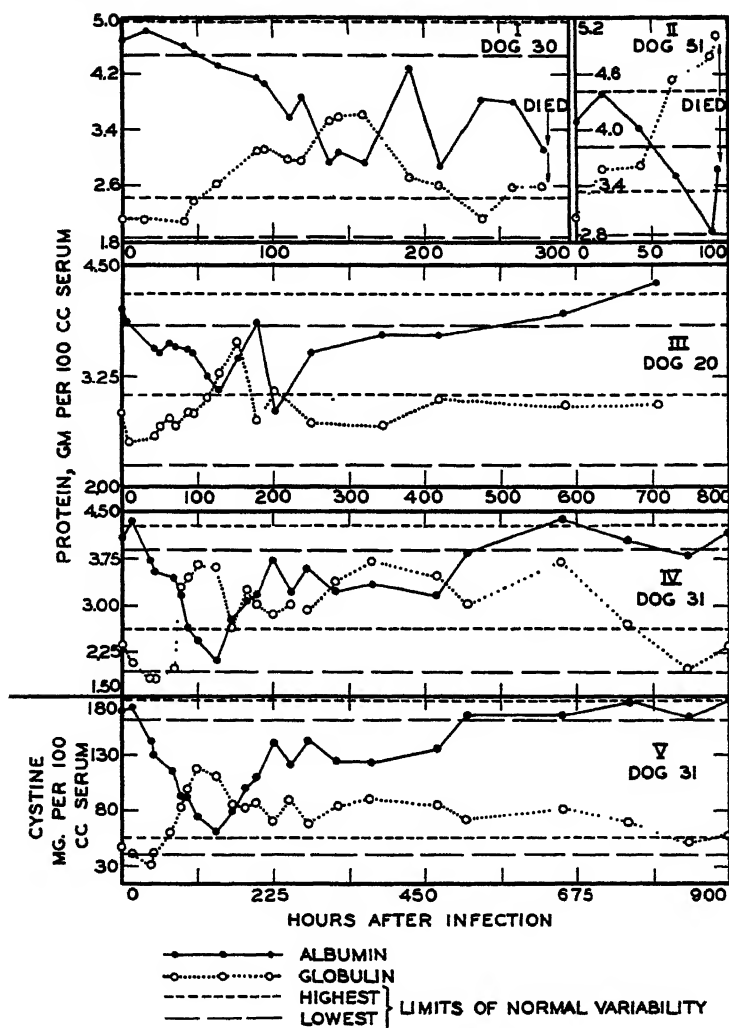


FIG. 1. Albumin and globulin concentrations of dog sera during type I pneumococcal pneumonia

made in clinical studies. In the four dogs the ratios from nitrogen data decreased from average normal values of 1.33, 1.40, 1.75, and 2.19 to

below 1; the lowest ones for each animal corresponding to 0.60, 0.94, 0.57, and 0.80, which occurred at the 90th, 130th, 140th, and 160th

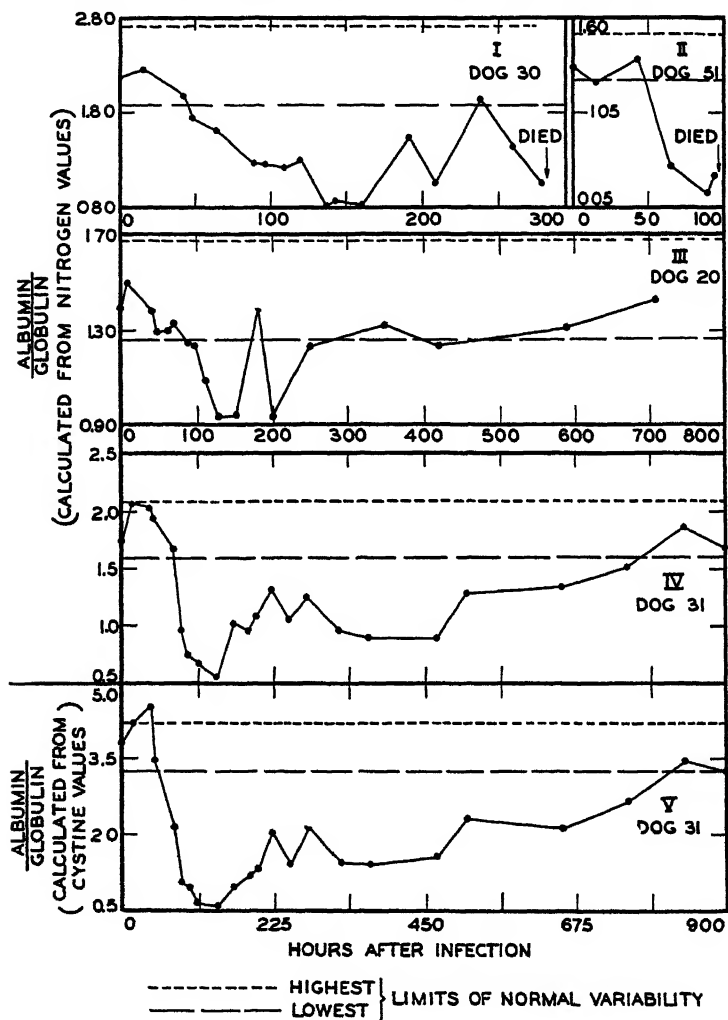


FIG. 2. Albumin/globulin ratios of dog sera during type I pneumococcal pneumonia

hours after infection, respectively. From the above average normal values of the ratios, it is seen that each animal appears to have an indi-

vidual, characteristic normal albumin and globulin concentration. The corresponding average ratios of the two other dogs not included in this discussion were 1.77 and 1.79, respectively. Similar ratios and similar individual variations for dogs have been reported in the literature (11, 12, 13). The ratios calculated from the cystine data were numerically 2 to $2\frac{1}{4}$ times greater than the corresponding ones from nitrogen analyses, but they also decreased to values below 1 during the course of the infection. The percentage decrease in the ratios based on cystine analyses was, therefore, considerably greater than in those calculated from nitrogen values. This is partially due to the assumption used in the calculations that each of the proteins contains 16 per cent nitrogen, while their cystine content is known to differ. Serum albumins precipitated once by the usual salting out methods have about twice as much cystine as the globulins. Any slight decrease in albumin concentration is therefore reflected more in the ratio calculated from cystine determinations, provided that neither the nitrogen nor the per cent cystine content of the two proteins changes during pneumonia. This latter assumption can be verified by calculation, for if no change in either composition occurs, then the following equation should hold throughout the course of the disease:

per 100 cc. of serum,

$$\frac{\text{mg. cystine found in albumin}}{\text{mg. cystine found in globulin}} = \frac{\text{mg. nitrogen found in albumin}}{\text{mg. nitrogen found in globulin}} \times k$$

or

$$k = \frac{\text{mg. albumin cystine}}{\text{mg. globulin cystine}} \times \frac{\text{mg. globulin N}}{\text{mg. albumin N}}$$

When k was calculated from the experimental data for each serum sample of each of the six dogs prior to their infection, average values of 1.32 (1.21–1.47), 1.65 (1.27–2.18), 1.69 (1.28–2.00), 1.79 (1.56–2.42), 1.83 (1.50–2.12), and 2.19 (1.80–2.64) were obtained, with the figures in parentheses representing the lowest and highest normal values, respectively, encountered for each dog. After the inoculation of the dogs with pneumococci the k values of the sera of the four dogs with definite pneumonia began to fall steadily, reaching values near 1 or below (0.87 as the lowest), and returning to normal upon recovery. In the case of the two dogs in which the infection did not take, a very slight fall below the lowest normal k value took place in one and none in the other. Any

consistent fall in k below that likely to be due to experimental error or to individual variability of the dog must reflect a change in either the nitrogen or cystine content of one or both of the serum proteins during the infection. Upon the premise that the nitrogen composition was more likely to remain constant, the cystine content per 100 g. of protein ($N_2 \times 6.25$) was calculated. During the course of the infection no change in the cystine composition of the globulins was observed. The average cystine values of the globulins of each of the six dogs, as well as the lowest and highest values found (in parentheses), were: 2.00 (1.73–2.32), 2.22 (1.73–2.41), 2.43 (1.75–2.90), 2.54 (2.01–3.03), 2.67 (1.83–3.24), and 3.08 (2.56–3.73). In contrast to this, the cystine concentration of the albumins decreased steadily during the periods of increasing consolidation of the lungs, and returned to normal upon recovery. These data are shown in Fig. 3, Curves I to IV, where g. of cystine per 100 g. of albumin is plotted against time after the inoculation of the pneumococci. The two broken, parallel lines again represent the minimum and maximum limits of normal variability for each dog. The extent of these fluctuations in normal values is undoubtedly due to a combination of experimental errors occurring during protein precipitations or nitrogen and cystine analyses, and it is quite reasonable to assume that the actual daily variability is much less than is apparent from the values represented by the two broken lines in the curves. Since during the course of the disease the per cent cystine content falls steadily, and in all but one case (Dog. 20) to a considerable extent below the lowest limit of previous experimental variation, it seems evident that there is a decrease in the cystine of the serum albumins of dogs during experimental pneumonia. It should be recalled in the case of Dog. 20 (Fig. 3), in which the cystine percentage decreased slightly and only for a short period of time, that this dog had a relatively milder infection than Dogs 30, 31, and 51. This is clearly shown in their respective albumin/globulin curves in Fig. 2, in which the ratios of Dog 20 returned to normal levels much sooner after infection than in the other dogs. The lowest per cent values to which the cystine content fell were 2.62, 2.88, 3.09, and 3.23 for the four dogs. As the average per cent cystine composition of once-precipitated albumins lies between 3.91 and 4.20, and since 3.64 per cent was the lowest value encountered in 51 analyses of normal dog sera, the consistently lower range of the cystine values during infection is significant. It is also interesting that, in the case of Dog 30, the animal which showed indications of recovery from the

pneumonia but finally died after the onset of jaundice, the per cent cystine content of the albumins returned to normal after the crisis was passed (Fig. 3, Dog 30), while the albumin-globulin ratio remained abnormal until the animal died (Fig. 2, Curve I). Thus, a low ratio is

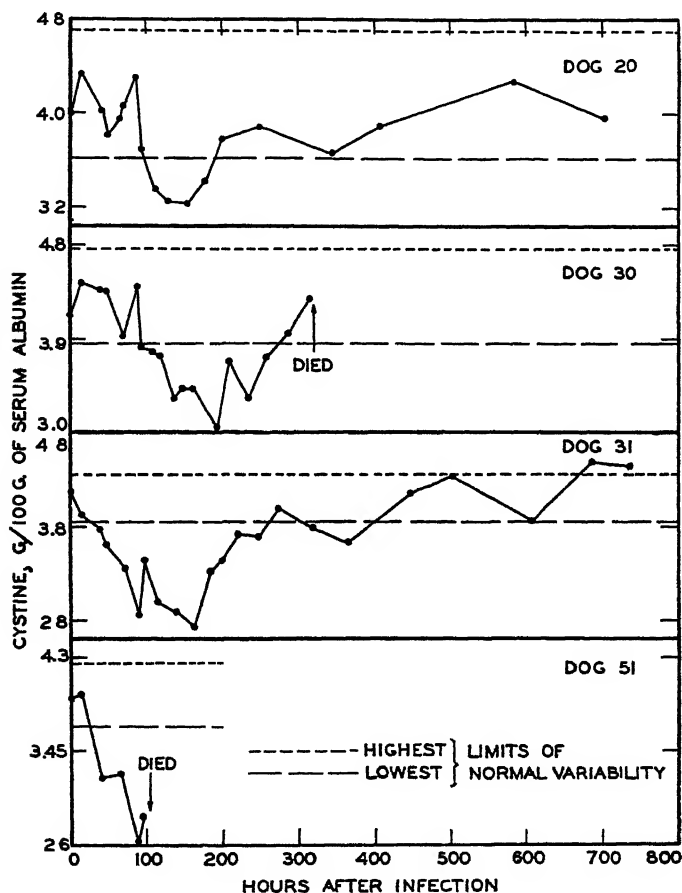


FIG. 3. Cystine content of dog serum albumin during type I pneumococcal pneumonia

not a prerequisite for a low cystine content of the albumin fraction of the serum. This observation should therefore eliminate any supposition that the low per cent cystine values of the albumins are due to the presence of abnormally large amounts of globulins which failed to be pre-

precipitated by the salting out procedure. It is felt, rather, that an albumin-like but cystine-poor protein forms during the course of the infection which is rapidly removed from the blood stream as recovery takes place. In this connection it should be recalled that Abernethy and Avery (14) and MacLeod and Avery (15) described a protein in the serum of humans and monkeys during the acute stages of certain infections, including pneumonia, which differed from normal serum albumin in its specific precipitation by the C polysaccharide of pneumococcus, but which, like normal albumin, was soluble at 50 per cent and insoluble at 75 per cent sodium sulfate saturation. Furthermore, Alving and Mirsky (16) reported the presence of an albumin in human cases of Bright's disease which contained less cystine than normally, but which, when dialyzed against distilled water, could be separated into a cystine-poor and a normal serum albumin. Previous studies from this laboratory established polarographically the presence of a low-molecular weight protein in the serum of dogs during the acute stages of pneumonia which differed from the peptone-like proteins of normal dog serum in its behavior towards alkali (17).

SUMMARY

Concentration changes and cystine percentages in the serum proteins of dogs were investigated in relation to time of infection with Type I pneumococcal pneumonia.

After the inoculation of the pneumococci, the protein level remained normal for a period of 50 hours. Thereafter, the albumin concentration fell gradually to a minimum of approximately one half of its normal value, while the globulin content of the serum increased, reaching a maximum concentration of almost twice that of the normal level. Consequently, an inversion of the albumin-globulin ratio usually took place at the height of the infection which occurred between the 90th and 160th hours after the inoculation of the pneumococci, depending upon the severity of the infection. In general the drop in the ratio is most sudden for the sera of dogs with the most severe cases of pneumonia, and the time necessary for a return to a normal ratio is very long, extending to over 700 hours in one of the dogs.

During the course of the infection, the cystine percentage of the globulins remained constant, while the cystine content of the albumins decreased steadily beginning with approximately the 50th hour after the infection. Low values of 2.62, 2.88, 3.09, and 3.23 g. of cystine per

100 g. of albumin were found in the case of four dogs with typical symptoms of lobar pneumonia. Upon recovery from the disease, the cystine content of the serum albumins returned to their previous normal levels of 3.91-4.20 per cent.

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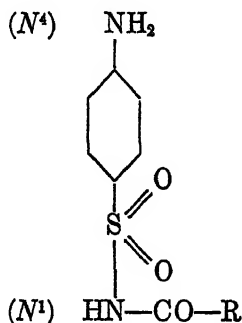
The Metabolism of N^1 -Acylsulfanilamides in the Dog

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The search for new chemotherapeutic agents of the sulfonamide type, which has been carried on during the past few years, has led to the synthesis of a series of N^1 -acyl derivatives of sulfanilamide (1). These are of the type



where R represents an alkyl group of from 1 to 11 carbon atoms. In some cases the isomeric as well as the normal groups have been used as substituents.

Since assays of the therapeutic activities of these compounds showed irregularities which could not be explained on the basis of what was then known about their absorption (2), a more extensive study of their metabolism was undertaken. It is with the results of this investigation that the present communication deals.

PROCEDURE

Dogs were used as experimental animals because of the fact that they do not acetylate sulfonamides in the N^4 position. In the experiments to be reported here, single doses of the drugs were administered,

usually 0.5 or 1.0 g./kg. of body weight. When administered orally, they were in the powder form contained in gelatin capsules. In two experiments they were injected intravenously as solutions of the sodium salts. These two experiments will be mentioned individually in the text. Following drug administration, the animals were placed in metabolism cages and their urine collected daily. Small samples were taken for analysis by the method of Bratton and Marshall (3), and the rest stored in the refrigerator. Collection was continued until 50–70% of the drug administered had been excreted. This insured a sufficient yield of drug to render purification and identification easy.

ISOLATION OF END PRODUCTS FROM URINE

1. *Sulfanilamide*

In the early experiments, the urines from all of the dogs which had received *N*¹-acylsulfanilamides were treated in the same way. The total urine from one dog (or if the volume was large, one liter of it) was extracted continuously with ether for 48–72 hours. The resulting ether extract was then evaporated to dryness, the residue taken up in a small volume of hot 50% ethanol, and the alcoholic solution placed in a refrigerator to cool. A good crop of yellow or brownish crystals usually resulted. These were filtered off, dissolved in warm 50% ethanol, and the solution shaken with successive small amounts of activated carbon (Norit A) until it was colorless. The colorless solution was then evaporated to small volume, cooled, and the resulting crystals filtered off and washed with a small volume of ice water. After drying, the melting point of the crystals was determined. Crystallization was repeated until a constant melting point was obtained.

By the use of this procedure, it was found that, after the oral administration of any of the *N*¹-acyls, with the exception of *N*¹-acetylsulfanilamide, sulfanilamide could be isolated from the urine.

2. *Other End Products*

As mentioned previously, sulfanilamide could not be isolated from the urines of dogs which had received *N*¹-acetylsulfanilamide. After long extraction (six to seven days) these urines yielded small amounts of the same drug which had been fed, *i.e.*, *N*¹-acetylsulfanilamide. The long extraction and low yields are the result of the low ether solubility of this compound.

The residues from these extractions were made up to approximately

1 N with solid NaOH, refluxed for five hours and finally neutralized with concentrated HCl. Re-extraction with ether then yielded sulfanilamide.

The foregoing experiments clearly demonstrate that, with the single exception of N^1 -acetylsulfanilamide, the drugs of this series are, partly at least, broken down to sulfanilamide in the body. In view of the irregular results of the therapeutic tests, however, it seemed important to find out whether any of the drug administered passed through the body unchanged. This question was answered by the use of a modification of the extraction technique already described.

In one type of preliminary experiment, samples of normal dog urine were made up to a known concentration with sulfanilamide, and in another with both sulfanilamide and N^1 -butyrylsulfanilamide. The solutions were then adjusted to pH 9-9.5 with NaOH. At this pH the sulfanilamide, being a very weak acid, will be present largely in the undissociated form which is ether-soluble, while the N^1 -butyrylsulfanilamide, a fairly strong acid (pK_a about 5.32) will be present as the anion of its sodium salt which is insoluble in ether. After adjusting the pH, the solutions were extracted continuously with ether. Samples of the residue were taken for analysis at approximately 24-hour intervals, and if the pH of the residue had fallen it was readjusted. Curve I, Fig. 1 shows the course of such an extraction when sulfanilamide alone was present in the urine, while Curve II shows the course when the urine contained both sulfanilamide and N^1 -butyrylsulfanilamide. It is clear that sulfanilamide is removed rapidly by this procedure, but when N^1 -butyrylsulfanilamide is present as well, the concentration of diazotizable material in the residue approaches asymptotically a value well above zero. Thus, in the experiment illustrated by Curve II, Fig. 1, the concentration fell rapidly until a value of 35 mg./100 ml. was reached and then, much more slowly, to 26 mg./100 ml. The concentrations are expressed as sulfanilamide. The concentration of N^1 -butyrylsulfanilamide originally present in this urine was 29 mg./100 ml., expressed as sulfanilamide. This agreement was interpreted as indicating that the diazotizable material remaining in the residue after ether extraction at pH 9-9.5 was N^1 -butyrylsulfanilamide.

When several days' extraction at pH 9-9.5 failed to reduce appreciably the concentration of diazotizable material in the urine to which N^1 -butyrylsulfanilamide had been added, the residue was removed from the extractor and its pH reduced to about 3.5 by the addition of HCl.

At this pH the *N*¹-butyrylsulfanilamide will be present in the undissociated form which is ether-soluble. When ether extraction was resumed, the remaining diazotizable material was rapidly removed (Curve II, Fig. 1). The same types of curves were obtained when other *N*¹-acyl derivatives were substituted for *N*¹-butyrylsulfanilamide.

A series of four such experiments were carried out and Table I shows the agreement between the *N*¹-butyrylsulfanilamide originally added to the urine and the approximate asymptote approached by the time-extraction curve.

Although this agreement left little doubt as to the identity of the diazotizable material remaining after extraction at pH 9, final proof came with its isolation. The pooled ether extracts obtained at pH 3.5 in three experiments were treated exactly as described previously for

TABLE I

Experi- ment	Sulfanilamide added mg./100 ml.	<i>N</i> ¹ -Butyryl- sulfanilamide added mg./100 ml.*	Asymptote of time extraction curve mg./100 ml.*
1	300	71	74
2	450	42.5	43
3	450	29	26
4	100	14.2	15

* Expressed as sulfanilamide.

the isolation of sulfanilamide. A melting point determination proved the product to be *N*¹-butyrylsulfanilamide.

When this procedure was applied to the urine of dogs which had received sulfanilamide, and of others which received *N*¹-butyrylsulfanilamide orally, the time-courses of extraction were similar to those of urines known to contain the drugs. Thus, Curve III, Fig. 1, shows the course of extraction at pH 9 of urine from a dog which received sulfanilamide orally. The drug was rapidly removed. Curve IV shows the course of extraction at pH 9 of urine from a dog which received *N*¹-butyrylsulfanilamide orally. This curve falls rapidly at first but approaches asymptotically a value of approximately 46 mg./100 ml., a figure which represents 10% of the total diazotizable material initially present in the urine. After acidification, further extraction removed the remaining diazotizable material rapidly. In five experiments, the approximately constant concentration of diazotizable material in the residue after

extraction at pH 9 amounted to 16% of that initially present in the urine. A crystalline product was isolated from the pooled acid extracts from these experiments, and a melting point determination proved it to be N^1 -butyrylsulfanilamide.

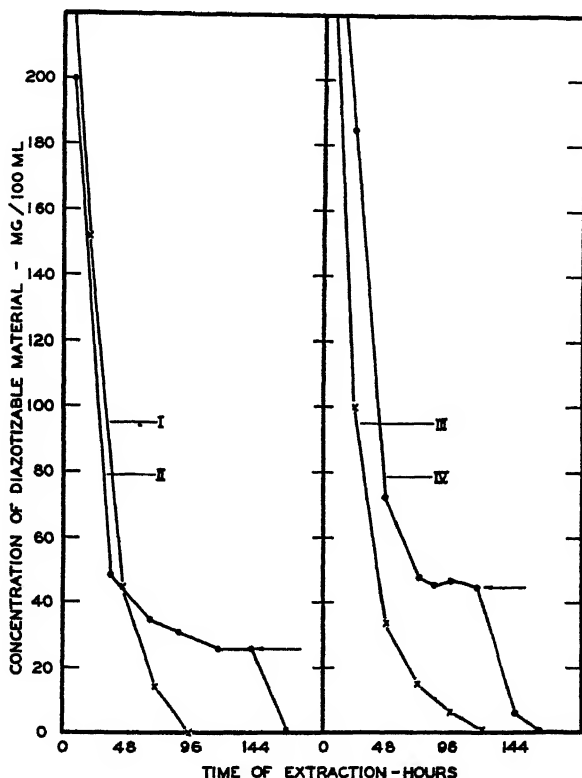


FIG. 1. Ether extraction, at controlled pH values, of dog urines containing sulfanilamide and N^1 -butyrylsulfanilamide.

Concentrations are expressed as sulfanilamide.

In II and IV, the pH values of the residues were lowered from 9 to 3.5 at the time indicated by the arrows.

Similar results were obtained in experiments in which dogs were fed N^1 -propionyl- and N^1 -valeryl-sulfanilamides. In three experiments in which N^1 -isobutyrylsulfanilamide was administered, however, an average of 35% of the drug excreted was unchanged.

In two experiments in which solutions of the sodium salt of *N*¹-butyrylsulfanilamide were injected intravenously, the fractions appearing in the urine unchanged were similar to those observed in dogs which had received the drug orally.

THE SITE OF BREAKDOWN OF *N*¹-ACYLSULFANILAMIDES IN THE BODY

Although the experiments in which sulfanilamide was recovered from the urines of dogs which received the sodium salt of *N*¹-butyrylsulfanilamide intravenously, show clearly that the drug can be split after absorption into the blood stream, there is still the possibility that some breakdown may take place in the digestive tract. This possibility was investigated by administering *N*¹-butyrylsulfanilamide orally to two dogs and sacrificing them after three and four hours when absorption was well under way. The gastric and intestinal contents of these animals were washed out separately with 50% ethanol and after filtration, suitable dilution and pH adjustment, the ultra-violet absorption spectra of the ethanol solutions were examined. The spectra were those of the drug which had been administered.

In another experiment small quantities of commercial pepsin, trypsin, and pancreatin were incubated at 37°C. in dilute solutions of *N*¹-butyrylsulfanilamide buffered to suitable pH values. Samples were taken at 12, 24, and 36 hours; and after suitable dilution and pH adjustment, their ultra-violet absorption spectra were obtained. There was no evidence of breakdown.

Since the splitting of the *N*¹-acylsulfanilamides occurs after its absorption into the blood stream, it was decided to attempt to determine the organs capable of carrying out this reaction by incubating tissue slices in solutions of *N*¹-butyrylsulfanilamide and then analyzing the solutions for sulfanilamide. Before these experiments could be carried out, it was necessary to develop an analytical method suitable for determining the relative composition of a mixture of sulfanilamide and *N*¹-butyrylsulfanilamide. The method finally used was spectrographic and depends upon the fact that each drug exhibits a single absorption maximum in the ultra-violet. Between pH values of 2.5 and 4.5, these maxima are of equal intensity but differ in wave length by 110 Å.¹ Mixtures of the two drugs also exhibit a single maximum lying between those of the pure compounds.

The ultra-violet absorption spectra of solutions of the pure compounds and eight known mixtures were obtained using a medium Hilger quartz

¹ Sulfanilamide—2590 Å. *N*¹-Butyrylsulfanilamide—2700 Å.

prism spectrograph with a Spekker photometer, and the positions of the absorption maxima accurately located with a Leeds and Northrup recording densitometer. These values for the wave length of the maxima were then plotted against relative concentration, giving a curve from which the percentage composition of an unknown mixture could be read after the position of its absorption maximum had been determined. It was found that the error of these determinations did not exceed $\pm 3\%$

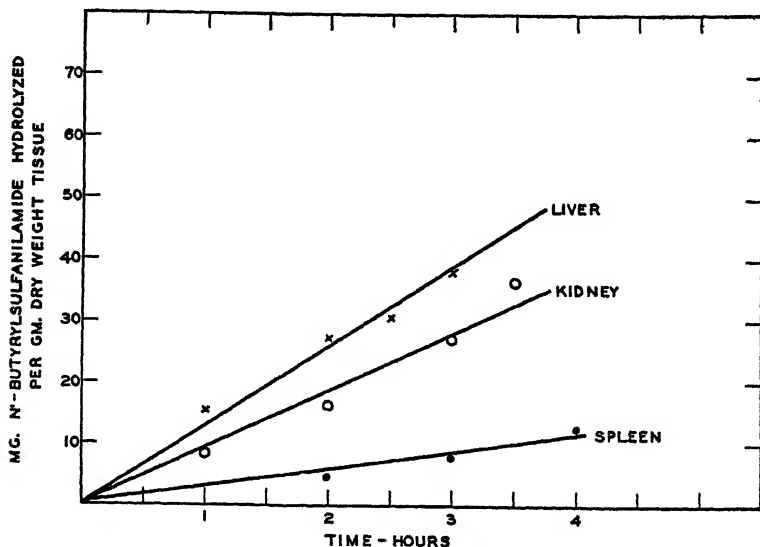


FIG. 2. Incubation of N^1 -butyrylsulfanilamide with rabbit tissue slices. Initial concentration of drug, 270 mg./100 ml.; temperature, $25^{\circ}\text{C}.$; pH, 7.2.

so long as the solution contained more than 10% of one compound in the presence of less than 90% of the other.

The incubation experiments were carried out as follows: To 20 ml. samples of N^1 -butyrylsulfanilamide solution (270 mg./100 ml. in 0.9% NaCl solution, buffered to pH 7.2 with phosphate) in 50 ml. Erlenmeyer flasks, were added thin slices of rabbit liver, kidney, spleen, heart, skeletal muscle, and intestinal muscle. To a seventh 20 ml. sample was added the washed red cells from 10 ml. of blood.² The tissues were

² Whole blood could not be used, for the plasma contains a substance absorbing so strongly in the ultra-violet as to obscure or seriously distort the absorption bands of the drugs. The same is true of urine, so that the method cannot be applied to these fluids.

incubated in these solutions at 25°C., with constant agitation, and samples withdrawn at regular intervals for analysis. At the end of the incubation, the tissue slices were removed from the flasks, blotted to remove adhering water, and dried to constant weight at 100°C.

The analyses showed that, when incubated with some of the tissues, the *N*¹-butyrylsulfanilamide was broken down to sulfanilamide. Since the total amount of drug initially present, the fraction hydrolyzed, and the dry weight of the tissue, were all known, the results could be expressed as milligrams of drug hydrolyzed per gram dry weight of tissue.

At the end of five hours of incubation with heart, skeletal muscle, intestinal muscle, and red cells, none of the drug, or at least not more than 5% of it, was hydrolyzed. The results of the incubation with liver, kidney, and spleen are shown in Fig. 2.

These results show that at least three organs in the rabbit contain an enzyme capable of splitting *N*¹-butyrylsulfanilamide. Similar observations have been made using the tissues of rats and dogs.

Other experiments are being undertaken with a view to purifying and characterizing the enzyme responsible for this breakdown and the results of these will be the subject of a subsequent communication. It can be mentioned at this time, however, that highly active concentrates have been prepared which are capable of splitting any of the compounds of this group with the exception of *N*¹-acetylsulfanilamide. Furthermore, the rate of hydrolysis increases with increasing length of carbon chain in the substituent fatty acid, and is less in isomeric than in the corresponding normal compounds.

SUMMARY

1. When *N*¹-acetylsulfanilamide is administered orally to dogs, it is excreted in the urine unchanged.
2. When other *N*¹-acylsulfanilamides are administered either orally or intravenously, a portion of the drug is broken down and excreted as sulfanilamide while another portion is excreted unchanged. When the drug administered is *N*¹-butyrylsulfanilamide, approximately 15% is excreted unchanged.
3. The splitting of the higher *N*¹-acylsulfanilamides does not occur in the digestive tract, but after the drug has been absorbed into the blood stream.
4. Liver, kidney, and spleen contain an enzyme capable of bringing about this reaction.

The authors wish to express their appreciation to Mr. R. D. Williams for carrying out the colorimetric analyses, and to Mr. David Richardson for his assistance with the spectrographic work.

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Iron Requirements of Heterotrophic Bacteria

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Little is known of the exact iron requirements of bacteria. Apparently the only quantitative data to be found in the literature are those of Burk (2) on the heterotrophic genus *Azotobacter*, and of Ruhland (6) on the autotrophic hydrogen bacterium *Hydrogenomonas pycnoticus*. This is easily understood when it is realized that a chemically defined medium containing the best reagent grade materials shows no iron deficiency when tested with one of the simple heterotrophs, e.g., *Aerobacter aerogenes*, and practically none when this medium is subjected to ordinary methods for the removal of traces of iron.

A nutrient medium "biologically free" of iron has been reported by Waring and Werkman (8). The purpose of this paper is to report results obtained in growth experiments with six species of heterotrophic bacteria.

EXPERIMENTAL

Details of the preparation of the iron-deficient medium were discussed in a previous publication (8). The constituents of the medium were freed from iron by a chloroform-8-hydroxyquinoline extraction which resulted in a medium containing an estimated biologically available iron concentration of 0.0007 to 0.003 part per million.

The basal medium contained the following.

Dextrose.....	1.0 per cent
$K_2HPO_4 \cdot 3H_2O$	0.4 " "
KH_2PO_4	0.1 " "
$(NH_4)_2SO_4$	0.1 " "
$MgSO_4 \cdot 7H_2O$	0.01 " "

Salt solution containing proper amounts of Zn^{++} , Mn^{++} , and Cu^{++} to give a final concentration in the medium of 0.01 p.p.m. each.

This medium produced normal growth of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and two species of *Aerobacter* when adequate iron was added. Suboptimal growth was obtained for *Escherichia coli* and *Serratia marcescens* when 0.05 per cent asparagine was added to the basal. The medium was inadequate for 15 other species which are known to grow with an ammonium salt or asparagine as nitrogen source.

The cultures used were *Aerobacter indologenes* 23B, *Aerobacter aerogenes* 174, *Pseudomonas aeruginosa* 2F3, *Klebsiella pneumoniae* P78, *Escherichia coli* E26, and *Serratia marcescens* 2G1, of the Iowa State College collection.

Stock cultures were carried on plain agar slants, thus permitting a determination of the iron needs of the normal untrained organism. The standard inoculum was prepared by removing growth from the surface of the stock slant with a platinum loop, and suspending it in a sterile 2 ml. triple-distilled water blank until a turbidity equivalent to a McFarland No. 1 standard was obtained. Sterile pasteur pipettes were used to add 1 drop of this suspension to each flask in the series. Each complete series was inoculated at the same time and in the same manner with the same pipette; the inocula were thus kept as equivalent as possible as evidenced by the duplicate cultures.

Cultural Procedure. All culture vessels were of pyrex glass which was found to give the same results as fused quartz ware. Erlenmeyer flasks of 250 ml. capacity, containing 100 ml. of medium and covered with 50 ml. beakers, provided the best arrangement for comparative series. The beakers served as an excellent substitute for cotton plugs, and no contamination was ever detected.

Sterilization was by autoclaving at 18 lb. for 20 min.; allowance was made for a 3 per cent loss in volume. Incubation was at 30°C. The incubation period necessary to produce maximal growth on the chemically defined medium was predetermined for each species. *Aerobacter* and *Klebs. pneumoniae* required 24 hours, *Ps. aeruginosa* 48 hours, *E. coli* 60 hours, and *S. marcescens* 72 hours. The cultures were shaken often during incubation to facilitate entrance of oxygen.

The most suitable method of measuring growth was found to be the turbidimetric method of Gates (5). This method, within the range used, was found by dry weight checks and serial dilutions of suspensions to be surprisingly accurate. The photoelectric colorimeter was not satisfactory because the iron-deficient cells were chalk white (porphyrin-deficient) and had absorption capacities differing from the normal buff-colored cells.

RESULTS

Preliminary experiments determined the approximate iron requirements of each species. Culture series were then assembled with the proper amounts of added iron to cover the entire effective ranges with the metal as the only known limiting factor. With *E. coli* and *S. marcescens* the basal medium itself was not entirely adequate for optimal growth. The results with these organisms are, therefore, not as striking as others. Nevertheless, the effective range of iron concentration with

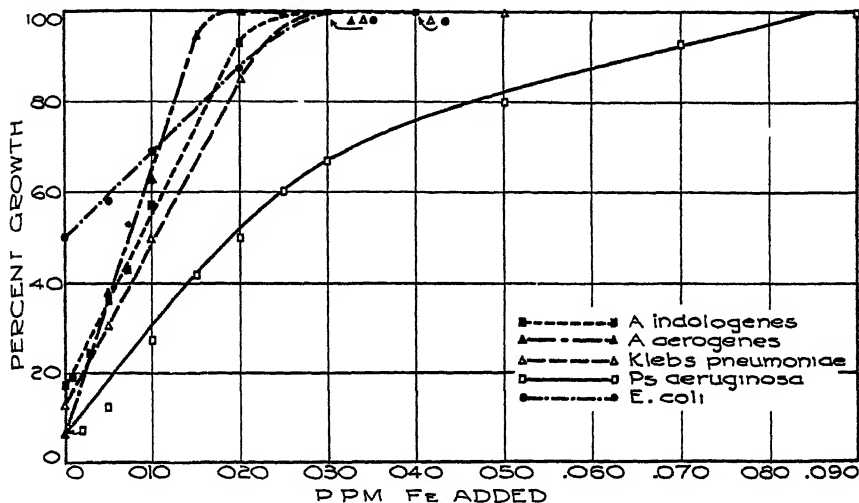


FIG. 1

these organisms appears to be clearly indicated, and is probably as valid as the others.

Aerobacter, *E. coli*, and *Klebs. pneumoniae* require an iron concentration of about 0.020-0.030 p.p.m. for maximal growth (Fig. 1). In each case the straight line indicates the simplest relationship between growth and iron concentration. With *Ps. aeruginosa* the curve formed may indicate a more complex relationship, possibly because this organism contains a complete cytochrome system. The results obtained with *S. marcescens* are the most difficult to explain (Table I). The percentage growth shown is calculated on the basis of the maximal turbidity being equal to 100 per cent, and the ratios were obtained by the method described by Gates.

The results of the culture series indicate the expected relationship between the iron requirements of the respective organisms and the types of iron-porphyrin systems they have been reported to possess. *Aerobacter*, *E. coli*, *Klebs. pneumoniae*, and *S. marcescens* each has an incomplete or primitive type of cytochrome system, Stephenson (7); Frei, Riedmüller and Almasy (4); and unpublished data. These organisms were found to have practically identical iron requirements for growth (0.020–0.030 p.p.m.), except that *S. marcescens* appears to have an additional iron

TABLE I
Relationship of Fe Content to Growth of Serratia marcescens

Culture No.	Fe added mg./liter	Turbidity reading	Maximal growth per cent	Prodigiosin produced
1	0	100	35	0
2	0.005	84	47	0
3	0.010	74	58	0
4	0.020	56	85	0
5	0.030	51	92	0
6	0.050	51	92	0
7	0.075	51	92	0
8	0.100	51	92	+
9	0.150	51	92	++
10	0.200	51	92	+++
11	0.300	48	97	++++
12	0.500	46	100	++++
13	1.000	46	100	++
14	2.000	46	100	++
15	3.000	46	100	0
16	5.000	46	100	0
17	10.000	50	94	0

mechanism associated with pigment production, which results in a slight increase in growth (Table I).

Ps. aeruginosa, on the other hand, possesses the complete 4 band cytochrome system and has higher catalase and peroxidase activities, also shows stronger hematin tests than those members of the former group for which data are available (Frei, *et al.*). Its iron requirement appears to be about 3 to 4 times that of the other organisms studied. It is unfortunate that sufficient growth was not obtained with some of the more distantly related species to allow for more information concerning the functional distribution of the iron within the bacterial cells.

The effect of iron on prodigiosin formation is interesting. This effect was noted by Bortels during his work with *S. marcescens* (1). In our studies with this species no pigment was formed in absence of iron and amino nitrogen. The habits of variation of this organism have been studied by many workers and have been found to be influenced by a wide variety of factors including carbon source, nitrogen source, mineral nutrition, certain wave-lengths of light, x-rays, and temperature of incubation.

Iron Content of Deficient Bacteria

Aerobacter indologenes was grown aerobically in the basal medium with increasing concentration of iron. The cells were carefully harvested,

TABLE II
Iron Content of Cells and Iron Content of Medium

Culture No.	Fe added <i>mg./liter</i>	Cells Fe content <i>per cent dry wt.</i>
1	0	0.0031
2	0	0.0036
3	0.010	0.0056
4	0.025*	0.0073
5	0.100	0.0196
6	1.000	0.0320
7	5.000	0.0902
8	10.000	0.1049

* Contained minimal concentration of iron for maximal growth.

washed twice with distilled water on an angle centrifuge and dried at 110°C. overnight. Samples of the dried bacteria were analyzed by a photometric modification of the thioglycolate method for total iron as described by Burmester (3). Table II shows the correlation of iron content of the cells and iron content of the medium in which they were grown.

In cultures 7 and 8, with a very considerable excess of iron, the cells also contained a considerable excess of iron. Although it is possible that the organisms may have the ability to store excess iron, most of the excess found was probably held by adsorption on the outer surface of the cell membrane. This adsorption may be easily demonstrated experimentally and will be discussed under iron availability.

Availability of Iron

Many workers have observed the apparent unavailability of iron that occurs occasionally in culture media, especially after heat sterilization. This phenomenon has not been adequately studied, with the result that the literature is confused concerning the cause. Apparently there are several factors operating. The medium may actually become temporarily iron-deficient for some species when the metal is precipitated from the medium, but this occurrence is probably rare. More important, and seemingly overlooked, is the colloidal interference that is possible when iron precipitation occurs.

With respect to the production of an iron deficiency, it may be said that this can occur only with those species which require a considerable amount of iron. The iron requirements of microorganisms are probably much more varied than is generally realized. The molds, because of their oxybiontic metabolism, require a considerable amount of iron for their iron-porphyrin systems and respiratory pigments such as aspergillin. They are also said to require iron for sporulation. The yeasts, when grown aerobically also require considerable iron for their strong cytochrome system. The bacteria appear to have a wide range of iron requirements. Some of the simple heterotrophs, as reported here, require very little of the metal, whereas others such as *Mycobacterium* appear to need relatively large quantities. It is quite possible that the precipitate formed in the medium will carry down with it by adsorption enough ionic iron to render the medium at least temporarily iron-deficient for the molds, yeasts, and a few of the bacteria; but this probably never occurs to the extent that organisms, such as the coli-aerogenes group, would be affected.

Experiments were carried out on the effect of autoclaving media containing different concentrations of FeCl_3 , FeSO_4 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and ferric citrate. In all concentrations below 2 p.p.m. there was no detectable difference between those cultures having iron added before or after autoclaving the medium. The test organisms used were those reported in the section on iron requirements. In all cases where the iron concentrations were raised to more than 3-4 p.p.m., there was a decrease in the amount of growth (Table III). This was the concentration at which the iron precipitated from the medium as hydrate. Removal of the precipitate from the medium by centrifugation or filtration resulted in full optimal growth, showing that the medium was not iron-deficient. When

citrate was added to the medium, precipitation of the iron did not occur until the concentration was well over 100 p.p.m. Decreases in growth were not observed with the citrate medium until the point of iron precipitation had been reached, showing that very large excesses of iron, when held in complex un-ionized form will not affect growth.

From the above experiments it was concluded that there must be an inhibitory effect on growth by the precipitate itself. This inhibition may be due to a transient colloidal adsorption on the cell wall, resulting in an interference with permeability. Since bacteria normally carry a negative charge, they will adsorb positively charged colloidal ferric hydrate. One gram of *A. indologenes* paste, when suspended in a freshly-prepared positive colloidal solution of ferric hydrate, adsorbed 1.18 g. of the col-

TABLE III
Effect of Excess Iron on Growth

	P P M Fe ADDED				
	10	20	30	50	100
<i>A. indologenes</i>	100	100	100	83	58
<i>A. aerogenes</i>	100	100	94	81	75
<i>E. coli</i>	100	100	96	89	85
<i>Klebs. pneumoniae</i>	100	100	90	75	55
<i>Ps. aeruginosa</i>	100	100	93	83	77
<i>S. marcescens</i>	100	100	100	92	87

Figures represent per cent of maximal growth.

loid. When potassium citrate was first added to the colloidal solution, the charge on the particles was reversed and no noticeable adsorption occurred on the cell walls.

The following simple experiment is of interest. A suspension of freshly harvested *A. indologenes* cells was divided into three equal parts. The first was treated with a freshly prepared positive ferric hydrate colloidal solution, the second with a negative ferric hydrate colloid, and the third remained untreated. The cells of these suspensions were then carefully washed and tested for oxygen uptake with glucose on the Barcroft-Warburg respirometer. The cells having the adsorbed positive iron colloid took up only 15 per cent of the oxygen that was taken up in unit time by each of the other suspensions. It appears evident, therefore, that a positive iron colloid, when present in a medium, may interfere with the general metabolism of the growing cell.

The availability of a number of highly insoluble iron compounds was tested in the iron deficient medium using *A. indologenes*. Powdered iron, FeCO_3 , Fe_2O_3 , FeS , $\text{Fe}(\text{OH})_3$, FeAsO_4 , $\text{Fe}_4(\text{Fe}(\text{CN})_6)_3$, and $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ were carefully washed, dried, weighed on a microbalance, and added to the purified medium to give concentrations of between 0.5 to 1.0 p.p.m. of Fe. Controls containing the same amounts of iron added as chloride attained normal maximal growth in 24 hours. The powdered iron produced normal growth as quickly as the chloride; the FeCO_3 , $\text{Fe}(\text{OH})_3$, FeAsO_4 and $\text{Fe}_4(\text{Fe}(\text{CN})_6)_3$ required 48 hours; the $\text{Fe}_4(\text{P}_2\text{O}_7)_3$

TABLE IV
Iron Content of Common Constituents of Media

Nutrient	Average iron content per cent
Bacto-peptone.....	0.0053
Bacto-dextrose	0.00014
Bacto-beef extract	0.0065
Bacto-yeast extract	0.0098
Bacto-agar	0.0016
Difco-chopped agar....	0.0077
Gen. Chem. Co. <i>d</i> -glutamic acid....	0.0009
Pfanstiehl C. P. <i>l</i> -asparagine	0.0006
Ether extract of Bacto-yeast extract* (100%).....	0.0003
Ether extract of Bacto-liver extract* (5%).....	0.0001
K_2HPO_4 Baker's Analyzed.....	0.0005
KH_2PO_4 Baker's Analyzed.....	0.0012
$(\text{NH}_4)_2\text{SO}_4$ Baker's Analyzed.....	0.00013

* Prepared by customary methods. The per cent strength of these extracts is on basis of original material.

and FeS , 60 hours; and the Fe_2O_3 , 72 to 90 hours. Evidently an organism requiring a large supply of readily available iron would not have obtained its needs from these sources, with the possible exception of the powdered iron; whereas *Aerobacter* is able after a period of time to dissolve enough iron, probably by producing organic acids, to meet its requirements.

Iron Content of Some Common Nutrients

During the course of this work, various nutrient materials were analyzed for iron. Data of this sort are lacking in the literature. Samples were taken in most cases from newly opened bottles, and were weighed

without drying or other treatment so as to simulate the ordinary handling practiced in preparation of culture media.

The results show why it is usually not necessary to add iron to a common laboratory medium. The traces of iron in these materials prove adequate. A medium containing as little as 0.1 per cent of the Bacto-yeast extract, or 0.2 per cent of the peptone or beef extract will supply the iron needs of most bacteria. The Bacto-agar, as it is used in most solid media (1.5–2.0 per cent) would supply 3 to 4 times the iron needed by most bacteria if it were all available.

SUMMARY

Exact iron requirements for growth of six species of bacteria (*Aerobacter aerogenes*, *Aerobacter indologenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Serratia marcescens*) in an iron-free glucose-(NH₄)₂SO₄-medium were determined. These requirements varied with the type of cytochrome system contained in the organism.

With *Serratia marcescens* production of the pigment prodigiosin was found to be a function of the iron concentration.

The iron content of cells grown in the presence of variable concentrations of iron salt was determined. *Aerobacter indologenes*, when grown without addition of iron contained 0.0031% Fe (dry weight basis). When grown with addition of its minimal normal requirement (0.025 p.p.m.) it was found to contain 0.0073% Fe. When grown with a large excess (10 p.p.m.) it contained 0.1049% Fe.

The availability of different soluble and highly insoluble iron compounds was determined.

Iron analyses of common constituents of culture media showed that the traces of this metal were of considerable magnitude and adequate for the bacteria used.

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Biochemical Lesions Produced by Diets High in Tyrosine

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The production of hypertension in rats fed large amounts of tyrosine has been reported (1). Histological examination of these rats (2) revealed perivascular fibrosis and arteriolar medial degeneration in various tissues and regressive and necrotizing changes in the parenchyma of pancreas and kidney. The symptomatology of the syndrome (1) and some of the histological findings (2) had been reported by Sullivan, *et al.* (3) and Lillie (4). The existence of a pancreatitis involving in its later stages the islets of Langerhans (2) and the edema and exudative keratitis (1, 3, 4) led to the assumption that biochemical lesions were probable.

EXPERIMENTAL

The diet used was a synthetic diet in which 10 per cent of the sucrose was replaced by 10 per cent tyrosine (1). On this diet, the rats develop a syndrome which runs a dramatic course, terminating in death in two or three weeks. The first indication is a blanching of the paws, which appear bloodless, a condition which it is felt corresponds to "blasser Hochdruck" (5). Then the paws become edematous and reddened; the edema extends to the head and shoulders. The animals assume a hunched posture and walk painfully. The cornea of the eye become opaque and an exudative blepharitis is seen in which the exudate is dark brown in color, suggesting melanin.

The initial studies were conducted on the blood sugar picture. Using the micromethod of Folin (6), blood sugars were determined at varying stages of the syndrome. During the first few days, the blood sugars of rats on these 10 per cent tyrosine diets are within the normal range of 75 to 113 mg. per 100 ml., averaging 98 mg. for 20 rats. At the stage in the syndrome characterized by the appearance of pale, anemic looking paws, "pale hypertension," the blood sugars are essentially normal;

but with the appearance of exudative blepharitis and reddening and edema of the paws, the blood sugars are markedly elevated, averaging 134 mg., within the limits of 50 and 205 mg. per 100 ml. Table I lists the blood sugar results obtained with the synthetic diet containing the tyrosine and various modifications and controls of that diet.

The blood sugar method used (6) gives values for reducing substances in the blood and are therefore high for glucose values. The values were checked using the method of Somogyi (12) which determines true glucose values through the use of fermentation procedures. A total of 20 tyrosine fed rats were used which permitted 10 determinations on a

TABLE I
Values for Blood Sugar in Rats Fed Tyrosine
(Values expressed in mg. per 100 ml. for blood sugar)

	Number	Blood Av	Sugar Max	Values Min.	Above Normal	Per cent Above Normal
Stock Diet	20	98	113	75	0	0
Synthetic Diet	10	100	115	80	0	0
Stock Diet plus 10 per cent Tyrosine	12	99	143	60	2	16
Synthetic Diet plus 10 per cent Tyrosine	40	134	205	51	21	52
Synthetic Diet plus 10 per cent Tyrosine and 2 per cent Ascorbic Acid	18	113	255	78	4	22
Synthetic Diet plus 5 per cent Tyrosine	18	99	160	62	4	22
Synthetic Diet plus 10 per cent Tyrosine until Symptomatic then on Stock Diet for 2 Mo.	5	68	74	60	0	0
Synthetic Diet plus 10 per cent Tyrosine and 1 g. Yeast Daily	9	115	133	88	5	55

sample of pooled blood from 2 rats. The results indicate that non-glucose reducing values ranged from 8 to 30 mg. in the presence of glucose reducing values as high as 400 mg. per 100 ml.

The minimum values (Table I) observed in any given series may be due to the moribund state of some of the rats. The numbers of rats in any series involving a modified tyrosine diet are not large enough to be conclusive but they do indicate that whereas yeast (1 g. daily) does not alleviate the syndrome, ascorbic acid (2 per cent) and the stock ration do alleviate the severity of the tyrosine poisoning. The fact that rats may be placed on tyrosine containing diets until they develop symptoms and then be placed on a stock diet until they seem to have recovered and are then normal with respect to their blood chemistry indi-

cates that all of the results may not be due to destruction of the islets of Langerhans which would surely be permanent but may be due to biochemical disturbance resulting from the presence in the system of high concentrations of tyrosine. Judgment on the actual basis of the hyperglycemia must be reserved for further investigation. It is not desired at present to attribute the results entirely to pancreatic destruction, although the evidence favors this view.

Contrary to the observations of Butts, *et al.* (7) homogentisic acid was not found in the urine of these rats. Vitamin C involvement seems indicated by the decreased incidence of hyperglycemia in rats fed ascorbic acid and tyrosine in their diets. The work of Sealock and his associates (8, 9) has clearly demonstrated an interrelationship between tyrosine metabolism and vitamin C nutrition.

Lipoid metabolism as reflected in free and total cholesterol was normal in the tyrosine fed rats as was nitrogenous metabolism as reflected in non-protein nitrogen and total protein.

DISCUSSION

Hyperglycemia produced by the inclusion of tyrosine at high levels in the diet of the rat may be due to one of several mechanisms: inactivation of insulin by blocking the functional hydroxyl of tyrosine in the insulin molecule; the production of large amounts of tyramine with resultant hyperglycemia; the production of adrenaline with resultant hyperglycemia; or the destruction of the islets of Langerhans (2), a mechanism which is the most probable. The basic biochemical lesion (1), it is suggested, is the decarboxylation of tyrosine to produce tyramine. This occurs in the kidney, the site of the greatest concentration of tyrosine decarboxylase (10). This local tyramine production causes a renal ischemia and sets into action the mechanism associated with experimental renal hypertension (11).

SUMMARY

A hyperglycemia, resembling diabetes, is produced by the inclusion of large amounts of tyrosine in the diet of the young rat.

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Growth Factors Controlling Tomato Stem Growth in Darkness¹

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In the greenhouse tomato stem growth is quite irregular even under constant temperature and humidity, due to the cessation of growth during exposure to strong light. Therefore, most following experiments, aimed to identify the internal growth factors, were carried out in darkness. This was possible after it had been found that the cessation of stem growth after keeping whole tomato plants for more than 24 hours in darkness can be overcome by application of sugar to the leaves.

EXPERIMENTAL

In all experiments, tomato plants ("San Jose Canner") were used grown in sand in 4 inch pots and watered daily with nutrient solution. Tomato seeds were germinated in flats in sand, and after three to four weeks, when the seedlings had developed their third or fourth leaf, they were transplanted to the pots. Three to four weeks later the plants were ready for the experiment.

Sugar

Preliminary experiments showed that it is possible to increase tomato stem growth in darkness by applying sucrose solution to the leaves of the plant. Control experiments, performed to establish this sugar effect on growth in darkness and to determine a satisfactory routine procedure for testing the effectiveness of growth factors other than sugar, are described in the following paragraphs.

1. Submerging of a leaf attached to the plant in a sugar solution was sufficient to cause stems to continue growth in darkness (see Tables

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I and III). About the same effect was obtained when the leaf floated on the solution as when it was submerged.

2. Growth of the stem was equally good when the leaves were kept submerged in a 10 per cent sucrose solution with a piece of wire screen,

TABLE I
Growth in mm./24 hrs.

	Number of leaves left on plant and placed in 10 per cent sucrose									
	4		3		2		1		0	
	42 hrs	52 hrs	42 hrs	52 hrs	42 hrs	52 hrs	42 hrs	52 hrs	42 hrs	52 hrs
In greenhouse in daylight										
Upper 30 mm. of stem.	7.6	12.8	6.0	9.8	4.1	6.5	2.1	4.4	0.4	0.6
Lower portion of stem (\pm 120 mm.)	3.4	3.1	2.2	1.7	1.9	2.2	0.5	0.8	0.3	0.1
Leaf growth	10.3	20.8	8.5	18.6	10.1	17.5	8.0	13.5	2.4	3.4
Total stem growth (mean of both periods)	13.7		10.0		7.4		4.3		0.7	
Total top weight per plant after 94 hrs.	835 mg.		623 mg.		587 mg.		360 mg.		46 mg.	
In darkroom										
Upper 30 mm. of stem.	14.0	22.5	13.4	19.5	5.5	9.2	3.2	2.4	0.0	dead
Lower portion of stem (\pm 125 mm.)	8.3	6.6	7.3	6.0	3.5	2.1	1.5	0.8	0.6	dead
Leaf growth	5.0	3.1	4.7	3.1	3.7	2.8	2.5	1.4	dead	dead
Total stem growth (mean of both periods)	26.0		23.4		10.3		4.0		0.3	
Total top weight per plant after 94 hrs.	92 mg.		92 mg.		61 mg.		38 mg.			

Growth of tomato plants of same age and size compared at same temperature (27°C.) and humidity (70 per cent) in complete darkness with occasional red light and in daylight. Plants transferred from greenhouse to the two conditions 22 hours before start of experiment. All but 4, 3, 2, 1 or 0 of youngest full grown leaves cut off. The leaves left put singly in vials with 30 cc. 10 per cent sucrose solution. Each figure mean of 6 plants.

which permitted ready diffusion of oxygen to the leaf (5.8 mm.), as when they were covered with a glass plate, which must have caused anaerobic conditions in the leaf (6.1 mm.). Controls, with leaves submerged in water grew 1.0 and 1.0 mm. in the same period.

3. The unimportance of an abundant oxygen supply for sugar uptake made it possible to enclose a whole leaf in a glass vial, which was filled with the sugar solution. The vials were supported in an upright position by tying them to small stakes which were set in the sand of the pot next to the plant. The leaves were carefully bent over and inserted, top first, into the vials. Thirty cc. vials were found to be very satisfactory.

4. Sucrose was more effective than glucose or mannose in its growth promoting effect (in one experiment, *e.g.*: tapwater 0.8 mm., sucrose 10 per cent 7.2 mm., glucose 5 per cent 4.5 mm.).

5. The optimal sucrose concentration was 10 per cent (15 per cent sucrose: 14 mm., 10 per cent: 20 mm., 5 per cent 7 mm., tapwater 2.7 mm.). This sucrose solution had the same suction force (or diffusion pressure deficit) as the leaf under ordinary growing conditions.

6. Higher sucrose concentrations, and also salts, had an initial osmotic effect on growth, which was decreased for the first hours. Afterwards the plants treated with sugar resumed growth, but those treated with salts (like Na_2SO_4) did not grow more than the controls treated with water.

7. Immersion of the younger, full grown leaves in sugar gave better growth than immersion of older leaves. For this reason all the older leaves were removed before the experiment was started, leaving only two or three fully expanded upper leaves. All but the smallest growing leaves (10-15 mm.) were also cut off, so that the growing region of the stem could be observed and measured (GR in Fig. 1).

8. As shown in the Tables I and III and Fig. 2 stem growth was proportional to the number of leaves immersed in sucrose. Consequently

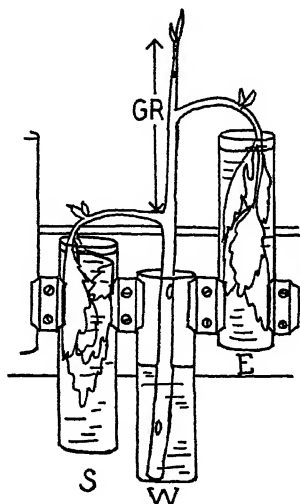


FIG. 1. Tomato shoot prepared for caulocline test. Cut stem placed in vial with water (W), one leaf in vial with 10 per cent sucrose (S), the other leaf in tube with extract to be tested (E). Measurements are made of the growing region (GR) between insertion of lower leaf and of youngest leaf, and of the youngest leaf length. The tubes are held by spring clamps on wooden cross bar.

it was not surprising to find that immersion in sugar of a single leaflet of the compound leaf was without measurable effect.

9. The greatest effects were observed in plants not over 25 cm. in length. In larger plants the growing point was often differentiated in a flower, and such plants showed a temporary decrease in growth rate until a new vegetative shoot developed from the axil of the highest leaf. In one experiment plants 40 cm. long grew 0.1, 1.0, and 0.9 mm. a day with 0, 1, and 2 leaves respectively, in sugar. Plants 25 cm. long grew 0.4, 3.2, and 8.6 mm. a day, and plants 15 cm. long grew 0.4, 2.6, and 6.3 mm. a day.

10. Perforating the leaf surface did not increase the effect of sugar. Perhaps the slight crushing of part of the leaf when inserted in the glass tube is necessary to get sufficient sugar uptake, but since this injury can hardly be avoided it has not been investigated.

11. If two leaves were left on a plant and one was immersed in sugar solution, it was immaterial for the ensuing growth whether the other leaf was left in air or submerged in water.

12. The conditions to which the plants were exposed before bringing them into darkness and treating them with sugar had an effect on their subsequent response, but this effect has not been systematically investigated. In individual experiments only plants of the same age and environment were compared.

13. When the older leaves were cut off when the plants were brought into the darkroom (generally 4:00 P.M.) they responded slightly less than when the leaves were left on during the 24 hours when the plants were kept in darkness before the experiment was begun.

In all experiments both stem and young leaf growth was measured. It was found that leaf and stem growth depended on different factors, and hence two sets of independent data were collected from each individual experiment.

The preceding control experiments were not reproduced in full, since the later, more elaborate experiments confirmed many of them and showed the feasibility of the technique elaborated in the earlier trials. Only a few experiments will be described in greater detail.

The first of these compares growth in darkness with growth in light. The plants in both conditions were comparable, since they had received the same pretreatment, and both sets had left 0, 1, 2, 3 or 4 leaves which were submerged in 10 per cent sucrose solution. Temperature and humidity were the same in the greenhouse and darkroom. In Table I

the growth in two successive periods of 42 and 52 hours is recorded. The growth of the individual internodes of the stem was measured, but to simplify the table only growth of the upper 30 mm. zone of the stem and the growth of the rest of the stem are presented. The growth of the stem was evidently proportional to the number of leaves immersed in sugar, so that apparently the amount of sugar taken up by the plant depended upon the surface of the leaves exposed to the sucrose solution. The growth of the young leaves was already maximal with two or three leaves in sugar, so beyond that, not sugar, but more specific leaf growth factors limited leaf development. Whereas, in daylight leaf growth increased in the second growth period, in darkness it dropped. This phenomenon supports the supposition that the other leaf growth factors

TABLE II

		Darkroom		Greenhouse		Greenhouse	
		2 leaves in		2 leaves in		2 leaves in	
		10 per cent		10 per cent		air	
		sucrose		sucrose			
		Stem	Leaf	Stem	Leaf	Stem	Leaf
Experiment 1	Dec. 5-8	4.7	2.3	5.6	13.2	2.1	7.3
Experiment 2	.. 11-13	3.8	2.8	3.4	8.5	1.5	6.8
Experiment 3	17-19	10.0	3.6	4.6	9.5	2.4	9.0

Comparison of stem and young leaf growth (in mm./day) in plants with two mature leaves. These are either left exposed to light in the greenhouse, or immersed in 10 per cent sucrose solution. The latter group is partly left in the greenhouse, partly brought in a darkroom with the same temperature (26.5°C.) and humidity (70 per cent).

are formed or activated in light but not in darkness (see Gregory, 1928, Bonner, Haagcn-Smit, and Went, 1939, Went 1941). When no sugar was supplied, the plants died after having been for over three days in darkness. Since the total stem growth rate did not decrease in darkness, except when not enough sugar was available, it was evident that the stem growth factors can be formed in darkness. Light has even a depressing effect on growth for when the same amount of sugar was supplied to plants in light and darkness stem growth was about twice as fast in darkness.

It is of interest to compare growth in light, when normal photosynthesis supplies the required sugar, and when the sugar is supplied from outside. In Table II some such experiments are summarized. They show that stem growth with 10 per cent sucrose was two to three times

faster than in plants with leaves exposed to light and air. Those latter plants grow very slowly since only two leaves were left. The normal growth rate of plants of that size having 6-8 full grown leaves is somewhere between six and ten mm. a day. Since the sugar content of these tomato leaves fluctuated between two and five per cent, the increased growth rate of the plants with leaves immersed in sucrose can be attributed to a larger sugar supply.

From these experiments it follows that both in light and in darkness sugar was limiting the shoot growth of tomatoes under the described experimental conditions, namely, plants grown in sand, watered with nutrient solution, and exposed to high temperatures and humidities. What, then, is the importance of the other stem growth factors in the tomato?

Auxin

First, auxin will be considered. In determinations of the auxin content of leaves, tips and stems of tomatoes grown in light or kept in darkness, no immediate decrease was found upon darkening. Even after three days in darkness, just before the plants started to die because of lack of carbohydrates, and long after growth in length had ceased, considerable amounts of auxin were extracted of the same order of magnitude as in the light grown plants. When the auxin and sugar experiments are considered together, doubt might be expressed as to the importance of auxin for regulation of stem growth in tomatoes. Earlier experiments had shown that auxin could modify growth of tomato stems (*e.g.* Hitchcock, 1935; Zimmerman and Hitchcock, 1938), but it had not been shown to be one of the essential growth factors. Therefore, an experiment was performed in which the growth of decapitated plants was compared with that of auxin treated and control plants. Young tomato plants, 20 cm. high, were brought at 4:00 P. M. into a darkroom. Twenty-four hours later, when their growth rate had dropped to less than 1 mm. a day, they were divided into nine groups of twelve plants each. Seven groups had only one leaf left on each plant. Since the experiment indicated that only the number of leaves submerged in sugar solution was of importance for the stem and leaf growth rates, the groups with one leaf and three leaves left were combined for calculation. Similarly the treatment of one leaf with a vitamin solution (10 mg. B₁, 10 mg. B₆, and 110 mg. of nicotinic acid per liter of water) did not have an appreciable effect on the subsequent growth of leaves and stems,

compared with similar plants in which this leaf was left untouched in air. The results are shown in Table III. As many other experiments showed (see *e.g.* Tables I and II) the behavior of these plants toward sugar was typical. Leaf growth was not increased very much when more than one leaf was submerged in sugar. That more sugar was taken up when more leaves were immersed was shown by the increased stem growth, which was proportional to the number of leaves in sugar. After the

TABLE III

	Period in hours after beginning of experiment	Three leaves in 10 per cent sucrose			Two leaves in 10 per cent sucrose; third leaf either in air or in vitamin solution			One leaf in 10 per cent sucrose; others cut off or left in air			No leaves in sucrose; 1-3 leaves left in air		
Number of plants per group		12			24			36			36		
Leaf growth in mm./day	0-42	6.0			4.3			4.3			0.0		
Top weight in mg. after 42 hrs.		290			233			228			92		
Stem growth in mm./day	0-42	14.6			7.3			5.1			0.6		
		intact	decap.	auxin	intact	decap.	auxin	intact	decap.	auxin	intact	decap.	auxin
Stem growth in mm./day	42-66	9.0	7.5	14.2	7.2	2.0	7.9	8.1	3.7	8.8	0.5	0.3	0.8

Growth measurements on tomato plants, grown in pots in ordinary greenhouse to a height of 20 cm. Experiment started 24 hours after transfer of these plants to a darkroom kept at 27°C. and 70 per cent humidity. All but 1 or 3 youngest full-grown leaves cut off. One, two or three leaves per plant submerged singly in glass vials with 30 cc. 10 per cent sucrose solution.

first period of 42 hours two-thirds of the plants of each group were decapitated, and half of these were treated with paste containing one part indole-3-acetic acid to 1000 parts lanolin. In the next 24 hours the plants which were left intact grew at approximately the same rate as in the previous period. In the decapitated plants the growth rate was reduced 50 per cent, but when they were treated with applied indoleacetic acid, they grew even more than the intact plants. This experiment

proves that auxin produced in the stem tips is just as essential for stem growth of tomato plants as it is in the *Avena* coleoptile and other stem-like organs. The factor limiting stem growth of tomatoes in darkness, however, is sugar.

Caulocaline

Growth of cut tomato stems falls off very rapidly, and after 24 hours the growth rate is usually well below 1 mm./day. Sucrose nor auxin can increase their growth beyond that rate. This is clearly shown in the experiment of Fig. 2. However, when roots developed on those cut stems, growth was resumed, which is shown in Table IV. A group of 30 tomato stems was cut and placed with their cut end each in a vial with

TABLE IV

Number of roots formed 7 days after cutting		Growth rate in mm./day for for the period (days after cutting)		
Spread	Mean	1-4	4-5	5-7
0	0	0.5	0.5	0.3
1-20	10	0.6	0.3	0.2
21-30	26	0.5	0.5	0.5
31-40	35	0.8	0.7	1.7
41-80	64	0.8	0.5	2.5

Growth rate of cut tomato stems placed in vials with water and having one or two leaves supplied with 10 per cent sucrose. Plants grouped according to the number of roots formed.

water (see Fig. 1). One or two leaves were left and placed in 10 per cent sucrose. Within a week 70 per cent of the plants had developed 2-74 roots on the portion of the stem submerged in water. The plants were grouped according to the number of roots developed 7 days after cutting the stems, and from Table IV it follows that the more roots were formed, the more pronounced the resumption of growth was. Less than 20 roots did not seem to affect growth at all, but beyond 30 roots the effect was very distinct.

This experiment confirmed the earlier conclusion that roots produce a growth factor necessary for stem growth (caulocaline, Went, 1938, 1939, 1942). Caulocaline was rapidly used up in cut stems, so that their growth rate dropped below 10 per cent of the original rate within 24 hours after cutting. Such cut stems, with at least one leaf in sucrose, became a very sensitive test object for caulocaline. Only upon supply

of the factor normally supplied by the root system would the cut stems resume growth.

The following routine method of caulocaline assay was found to be satisfactory, making possible an independent assay for leaf growth factors

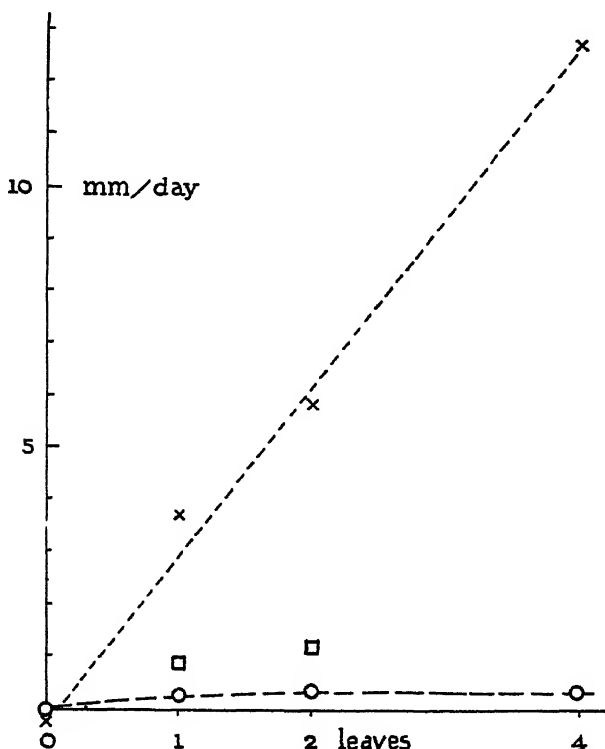


FIG. 2. Growth rate of tomato stems in darkness at 25°C. in mm. per day (ordinate, as mean of 6 plants each over a two-day period), after an initial period of 42 hours in darkness. Abscissa: number of leaves in 10 percent sucrose solution. Crosses refer to growth of intact plants with root system, circles and squares to cut stems (without roots). Squares represent growth of stems with an additional leaf in an extract containing caulocaline.

at the same time: Four weeks old tomato plants were cut from the roots and transferred to a dark room at constant temperature (25°C.) and high humidity (60–80 per cent). After 15–20 hours all the leaves except the two youngest fully expanded leaves, and the youngest apical leaf of ca. 15–10 mm. in length were cut off. The plants were then mounted in

racks in such a manner that the basal cut stem end was immersed in a 20 cc. vial containing water. The two leaves were each carefully stuffed into similar vials (see Fig. 1). One vial was filled with a 10 per cent sucrose solution, and the other with a solution of extract or substances to be tested. The length of the stem from the first internode to the apical internode was measured at 24 hour intervals. At the same time

TABLE V

Effect of Coconut Milk on Stem Growth in Cut Tomato Stems. One Leaf Submerged in Sugar (or Water), the Other in the Extract to be Tested.

Leaf		Growth in mm.		
First	Second	First day	Second day	Total in 2 days, individual plants
Water	Water	0.0	dead	0, 0, 0, 0, 0, 0
Sugar	Water	2.7	0.3	2, 3, 3, 3, 3, 4
Sugar	Coconut milk undiluted	4.2	2.9	3, 5, 6, 6, 9, 13
Sugar	Coconut milk diluted half	4.5	3.1	1, 2, 4, 9, 10, 20

TABLE VI

Caulocaline and Leaf Growth Activity of Natural Products as Measured by the Tomato Test

Activity is expressed as growth relative to sugar controls. Control = 100

Substance	Caulocaline Activity	Leaf Growth Activity
Coconut Milk.....	230	150
Pea Diffusate.....	230	170
Yeast Extract.....	130	200
Ground Pea Seed Extract (Water Soluble).....	100	—
Dried Tomato Leaf Extract (Water Soluble).....	100	—
Fresh Cow Urine.....	100	— *
Tomato Root Extract.....	100	—
Bean Sprout Extract.. . . .	100	—
Turnip Root Extract...	100	—
Control.....	100	100

* Leaf growth not quantitatively determined, but showed very marked stimulation.

the length of the apical leaf was measured. The difference in stem or leaf length as measured during a 24 hour interval was then the measure of growth. Application of sugar or of growth factors through the cut end was hardly effective, only when applied to leaves a response resulted. With one leaf submerged in sucrose already the maximal growth rate was reached, and immersion of more leaves in sucrose did not materially increase growth of cut stems (see Fig. 2.).

In Table V the effect of the immersion of one leaf in coconut milk is shown. Whereas already the first day a significant increase in growth rate of the stem occurred, when compared with the controls, the difference during the second day was much more remarkable. When these figures are compared with those of Table IV, it will be seen that the applied extract had about the same effect as the development of abundant new roots. This is another indication that the coconut milk supplied the same factor which normally the roots produce, *viz.* caulocaline.

The technique used allows the carrying-out of this test without aseptic precautions. Although the extracts tested are generally rich in bacterial

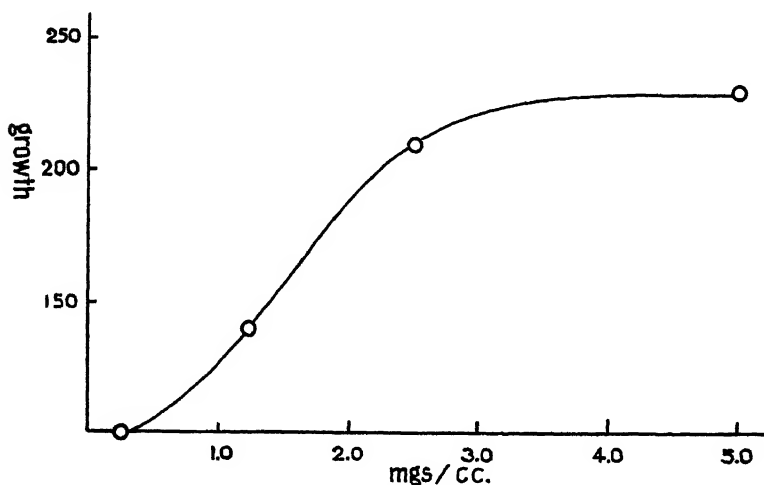


FIG. 3

growth factors, no marked decomposition occurs in the first 1-2 days, since the sugar is supplied separately to another leaf. The food and the growth factor supply are spatially separated, so that only inside the plant they combine, when bacterial action is excluded.

Experiments on the occurrence and identity of caulocaline were carried out using this test, or in some instances the tests were made using plants with intact root systems. Table VI lists different crude materials, and extracts tested for stem growth activity. Of these only coconut milk and pea diffusate (for method of preparation see Bonner, Haagen-Smit, and Went, 1939) were active. The activity of pea diffusate plotted in terms of growth above that of the sugar controls is shown in Fig. 3.

Thiamin, riboflavin, pyridoxin, inositol, nicotinic acid, pantothenic acid, and adenine were tested and found to be without activity. The only pure substance tested and found to be active was potassium nitrate. That it is identical with caulocaline seems unlikely since it has activity only at relatively high concentrations (maximum activity attained at a concentration of 5 grams/liter), and its activity computed in terms of total growth is only half that of the most potent extract, *viz.* coconut milk.

Since caulocaline is known to be made in the roots, many attempts were made to prepare an active extract of tomato roots. The root extract was prepared by freeing the roots of sand, and grinding them in water using a Waring blender. This method renders the roots as a very fine mash. This mash tested directly, or after filtering the water extract free of solid matter was inactive. Preliminary soaking of the roots in ether, or grinding them in methyl alcohol did not yield active extracts, nor did other root systems prepared in similar fashion.

As previously mentioned, there is good proof that the factors limiting stem and leaf growth are distinct entities. The sharp distinction between these two sets of factors can be clearly shown in this test. Pea diffusate, yeast extract, and cow urine are known to be potent sources of leaf growth activity (Bonner, Haagen-Smit, and Went, 1939; Bonner, 1940). Whereas pea diffusate possesses stem growth activity, yeast extract and cow urine do not increase stem growth although they increase leaf growth in the same test plants. Adenine has been recognized as a leaf growth factor (Bonner and Haagen-Smit, 1939; Bonner, 1940). Its activity in leaf growth was further substantiated by its activity in this test, yet it was found in the same test to be inactive in stem growth. It seems clear, therefore, that not only are the earlier observations on the identity of leaf growth factors corroborated by this test, but that the factors limiting stem growth are not the same as the factors limiting leaf growth.

DISCUSSION

In this paper it has been shown that tomato stem growth is determined simultaneously by (1) sugar, (2) auxin and (3) caulocaline. In general the sugar supply is limiting stem growth, but after decapitation the auxin supply becomes insufficient and growth drops to less than half the original rate. This drop can be completely overcome by application of indoleacetic acid. When the roots have been cut off, growth drops to

a mere fraction of its original rate, even though sugar and auxin are supplied in optimal amounts. Either development of new roots or the application of certain extracts (pea diffusate and coconut milk) can increase the stem growth rate, showing that the extracts contain the growth factor, caulocaline, normally supplied by the roots. This observation forms the basis of a bio-assay for caulocaline, which has been described in the previous paragraphs. In previous trials it had not been possible to demonstrate the existence of caulocaline outside the living plant. This lack of success has to be attributed mainly to a wrong method of application of the extracts.

The limitation by sugar of growth and developmental processes in plants lacking of sugar supply was demonstrated earlier (Bouillenne, 1933; Went, 1934; Bausor, 1942). The present paper is also a complete confirmation of many phenomena recently described by Spoehr (1942). He was able to make albino maize plants develop almost to maturity by supplying a 10 per cent sucrose solution to the leaves. Attempts to feed the sugar to the roots were not successful. In light leaf development was good, but stem growth was less than in darkness. In darkness with occasional weak light sufficient amounts of growth factors were formed to cause considerable growth in the presence of sugar.

Identification of caulocaline is impossible as yet and must await its isolation by a test method as described above. Its properties were discussed in earlier papers (*e.g.* Went, 1939) and so far no known substance was found to exert the same effect. It should be emphasized that most substances increasing stem growth have been tested on rooted plants, in which caulocaline is formed in abundance and therefore these tests could not detect caulocaline.

SUMMARY

In tomato plants grown in darkness, after an initial increase, stem growth drops to almost zero. This is due to a lack of sugar, which could be made up by applying a 10 per cent sucrose solution to immersed leaves. The growth rate of the stem was proportional to the number of leaves submerged in 10 per cent sugar, and exceeded growth of comparable plants with their leaves left exposed to light.

Auxin was limiting growth only after decapitation of the stem, as proven by restoration of the original growth rate in decapitated plants treated with indoleacetic acid.

Caulocaline was limiting the growth rate within 20 hours after cutting

the roots off. In special experiments it was confirmed that roots form caulocaline. A technique has been described which allows the testing of extracts and substances for caulocaline activity. Coconut milk and pea diffusate were found to contain caulocaline, whereas most known plant hormones were completely inactive.

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The Dissociation of Certain Amino Acids in Dioxane-Water Mixtures¹

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The effect of a particular solvent upon the titration behavior of a solute depends in part on the dielectric constant of the solvent. Most studies of dissociation constants have been carried out on aqueous solutions. However, a few studies in which solvents of lower dielectric constants than that of water were used have been reported (1). Jukes and Schmidt (2) determined the dissociation constants of a number of representative amino acids in ethanol-water mixtures. In confirmation of the earlier work of Mitzutani (3), they found that the chief effect of the ethanol-water mixture as contrasted with that of water is to decrease the dissociation of the carboxyl group while the dissociation of the amino group is affected to a lesser extent. Edsall and Blanchard (4) concluded from their studies that the zwitterion form of the amino acid exists in large amount even when the concentration of ethanol is as high as 86 per cent. Titration of amino acids in ethanol-water, in acetone-water, and in acetic acid (6) as a method of analysis has been used by various workers.

Due to the low dielectric constant of dioxane, mixtures of dioxane and water have been employed for studies on the behavior of electrolytes (7), (8-12). The feasibility of carrying out potentiometric titrations in dioxane-water mixtures of high dioxane content has been demonstrated by Lynch and LaMer (13) and by Gale and Lynch (14). Dielectric constants of dioxane-water mixtures are given by Robinson and Harned (12).

It was the purpose of the present investigation to determine the dissociation constants of representative amino acids in a solvent of lower dielectric constant than that of water. Mixtures of 20 per cent (weight per cent) of dioxane and water were employed both since the amino acids

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used are appreciably soluble in this solvent and since comparison could be made with certain studies carried out by Robinson and Harned (12) in this solvent.

EXPERIMENTAL

All materials used were of a high degree of purity as shown by analyses or were subjected to further purification. The titration of the amino acids was carried out with the aid of E.M.F. cells of the type

Glass	Amino Acid	KCl	HgCl, KCl (satd.), Hg.
electrode	HCl or NaOH	satd.	
	20 per cent		
	dioxane or water		

A Beckmann "high pH" glass electrode (National Technical Laboratory No. 1190-E) with its own length of shielded cable was employed. In titrating aqueous solutions of amino acids, the glass electrode was kept in a pH 10.0 buffer solution when not in use. Similarly, when the glass electrode was used for titrations in dioxane-water mixtures, it was kept in an alkaline solution of the same solvent when not in use. Electrodes previously used in water should be equilibrated at least 48 hours in the dioxane-water mixture before use. Depending upon the nature of the titration, HCl or NaOH dissolved in water or in 20 per cent dioxane was employed.

The titrations were carried out in a vessel similar to that described by Goyan and coworkers (15). The cross section of the vessel was more of the shape of an inverted cone so that 10-25 ml. of solution could be titrated or single measurements on 3 ml. of solution could be made. The titration chamber and the KCl reservoir were separate vessels, the KCl bridge of each being connected by a section of rubber tubing. A piece of string was led from the reservoir through the rubber tubing in order to lower the resistance of the circuit especially since the rubber tubing was compressed by a clamp during a determination. The stopcock at the bottom of the titration chamber was then kept open. The liquid junction was made in the 2 mm. glass tubing below the bottom of the titration vessel. This protected the stopcock grease from the dioxane solution. The calomel electrode was the conventional type with an auxiliary KCl reservoir.

All parts of the cell were maintained in an air bath at 25°. In addition, water at 25° was circulated through the jacket of the titration vessel. The dioxane-containing solution to be titrated was stirred by a stream of nitrogen previously passed through a 20 per cent dioxane-

water mixture. The potential measurements were made with the aid of an amplifier of the type described by Goyan and coworkers (15) and a Leeds and Northrup student-type potentiometer. Special precautions were taken to prevent leakage of current in the circuits.

The glass electrode was standardized by taking an empirical pH value of 4.65 for the 0.02 *M* solution of potassium acid phthalate in 20 per cent dioxane. On this basis, solutions containing 20 per cent dioxane and 0.1 equivalent of HCl per liter were found to have a pH of 1.10. The glass electrode gave reproducible potentials in 20 per cent dioxane solutions. This is at variance with the ideas of Dole (16) who states that the glass electrode cannot be employed in solvent-water mixtures. No ambiguous behaviour of the glass electrode was noted so long as precautions were taken to keep it equilibrated with the medium to be titrated and to wash out the titration chamber with portions of 20 per cent dioxane after each titration. The electrode was never in contact with pure water between measurements. The shift shown by Dole to occur in alcohol-water mixtures is apparently due to a change in the asymmetry potential of the glass electrode since the electrode was not standardized with a known buffer in each medium used. In preliminary experiments in which electrodes of Corning 015 glass were used, reproducible measurements were obtained in 40 per cent dioxane after standardizing at an arbitrary value for the pH of a 0.02 *M* phthalate buffer in that medium. The fact that the glass electrode will give accurate pH readings in solvent mixtures is not in accord with the theory proposed by Dole (16, pages 276–279) that the electrode mechanism involves migration of H_3O^+ through the glass membrane. Hubbard, Hamilton, and Finn (17) by interferometric methods found that those agents (acids, MgSO_4 , etc.) which were shown by Dole to cause "errors" in pH readings also inhibited swelling of the glass membrane surface. Dole interpreted this as indicating migration of hydrated protons through the glass. However, it is possible that a certain amount of water is required in the matrices in order to increase the surface of the glass in intimate contact with the solution. The experience of Bennett and coworkers (18), Davies and Addis (19), and Davies (20) with the glass electrode in ethanol-water mixtures is indicative of the reliability of this electrode under such conditions.

The titration curves of typical amino acids and certain related compounds in aqueous solution and in 20 per cent dioxane are shown in Figs. 1–9. The apparent dissociation constants are given in the legends to the figures.

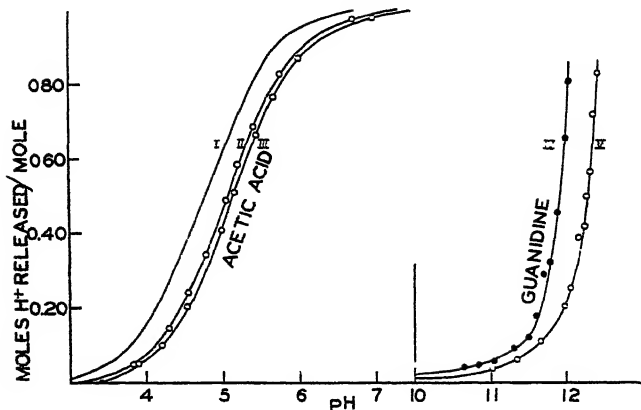


FIG. 1. TITRATION CURVES OF ACETIC ACID AND GUANIDINE IN WATER AND IN 20 PER CENT DIOXANE

I. Theoretical titration curve of acetic acid in water. $pK = 4.756$ (21). II. $0.0986\ M$ acetic acid vs. $0.476\ N$ NaOH (in 20 per cent dioxane). $pK' = 5.03$. III. $0.0197\ M$ acetic acid vs. $0.100\ N$ NaOH (in 20 per cent dioxane). $pK' = 5.13$. IV. $0.0199\ M$ guanidine sulfate vs. $0.1055\ N$ NaOH (in water). $pK' = 11.90$. V. $0.0200\ M$ guanidine sulfate vs. $0.1055\ N$ NaOH (in 20 per cent dioxane). $pK' = 12.48$.

Note: Curves IV and V begin a short distance above the base line due to the presence of a small amount of free acid as an impurity.

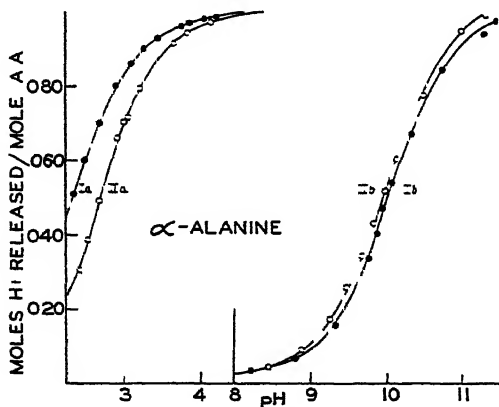


FIG. 2. TITRATION CURVES OF α -ALANINE IN WATER AND IN 20 PER CENT DIOXANE

Ia. $0.100\ M$ α -alanine vs. $0.1003\ N$ HCl (in water). $pK_1' = 2.47$. Ib. $0.0291\ M$ α -alanine vs. $0.0982\ N$ NaOH (in water). $pK_2' = 9.98$. IIa. $0.0359\ M$ α -alanine vs. $0.1020\ N$ HCl (in 20 per cent dioxane). $pK_1' = 2.70$. IIb. $0.0232\ M$ α -alanine vs. $0.1000\ N$ NaOH (in 20 per cent dioxane). $pK_2' = 9.93$.

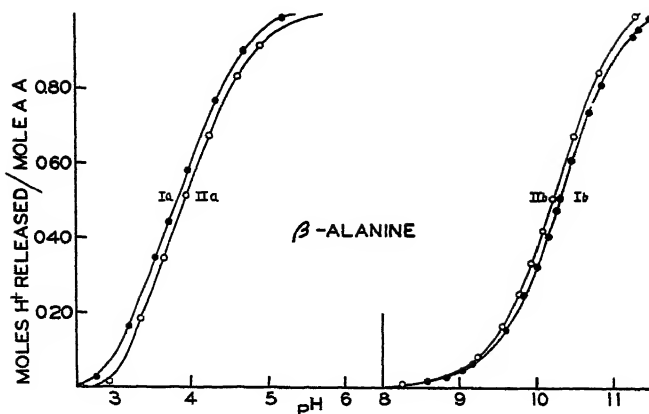


FIG. 3. TITRATION CURVES OF β -ALANINE IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0400 M β -alanine vs. 0.1024 N HCl (in water). $pK_1' = 3.65$. Ib. 0.1000 M β -alanine vs. 0.0982 N NaOH (in water). $pK_2' = 10.29$. IIa. 0.0239 M β -alanine vs. 0.0970 N HCl (in 20 per cent dioxane). $pK_1' = 3.82$. IIb. 0.0239 M β -alanine vs. 0.1000 N NaOH (in 20 per cent dioxane). $pK_2' = 10.22$.

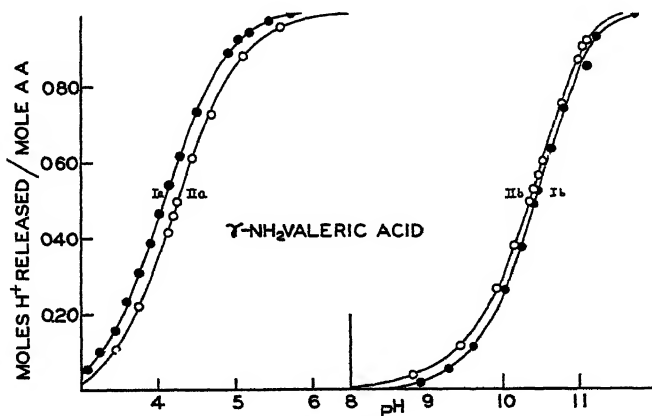


FIG. 4. TITRATION CURVES OF γ -AMINO- n -VALERIC ACID IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0130 M γ -amino- n -valeric acid vs. 0.1003 N HCl (in water). $pK_1' = 4.13$. Ib. 0.0130 M γ -amino- n -valeric acid vs. 0.0490 N NaOH (in water). $pK_2' = 10.43$. IIa. 0.0130 M γ -amino- n -valeric acid vs. 0.0511 N HCl (in 20 per cent dioxane). $pK_1' = 4.26$. IIb. 0.0130 M γ -amino- n -valeric acid vs. 0.0491 N NaOH (in 20 per cent dioxane). $pK_2' = 10.36$.

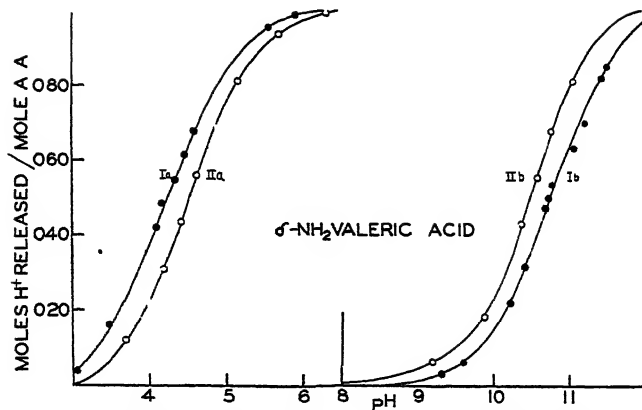


FIG. 5. TITRATION CURVES OF δ -AMINO-*n*-VALERIC ACID IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0156 *M* δ -amino-*n*-valeric acid vs. 0.1000 *N* HCl (in water). $pK_1' = 4.23$. Ib. 0.0156 *M* δ -amino-*n*-valeric acid vs. 0.0491 *N* NaOH (in water). $pK_2' = 10.70$. IIa. 0.0080 *M* δ -amino-*n*-valeric acid vs. 0.0501 *N* HCl (in 20 per cent dioxane). $pK_1' = 4.53$. IIb. 0.0080 *M* δ -amino-*n*-valeric acid vs. 0.0491 *N* NaOH (in 20 per cent dioxane). $pK_2' = 10.47$.

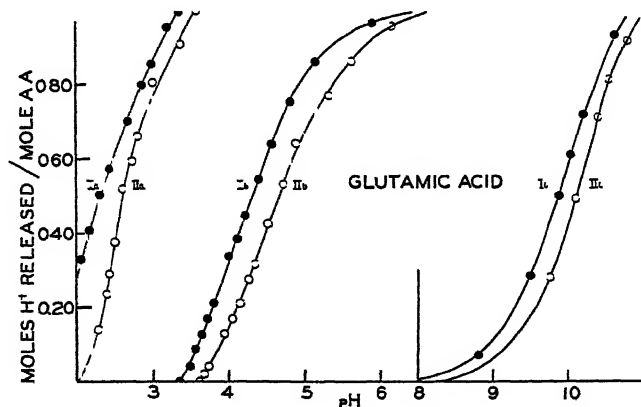


FIG. 6. TITRATION CURVES OF GLUTAMIC ACID IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0100 *M* glutamic acid vs. 0.100 *N* HCl (in water). $pK_1' = 2.35$. Ib. 0.0100 *M* glutamic acid vs. 0.1055 *N* NaOH (in water). $pK_2' = 4.33$. Ic. Continuation of curve Ib. $pK_3' = 9.88$. IIa. 0.0100 *M* glutamic acid vs. 0.0970 *N* HCl (in 20 per cent dioxane). $pK_1' = 2.60$. IIb. 0.0100 *M* glutamic acid vs. 0.1055 *N* NaOH (in 20 per cent dioxane). $pK_2' = 4.66$. IIc. Continuation of curve IIb. $pK_3' = 10.13$.

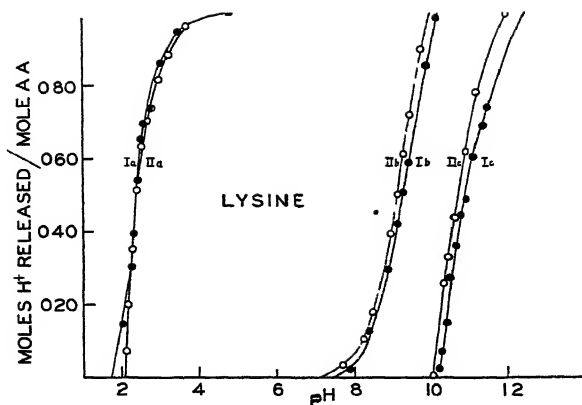


FIG. 7. TITRATION CURVES OF LYSINE MONOHYDROCHLORIDE IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0232 *M* lysine monohydrochloride vs. 0.1003 *N* HCl (in water). $pK_1' = 2.20$. Ib. 0.0232 *M* lysine monohydrochloride vs. 0.0982 *N* NaOH (in water). $pK_2' = 9.15$. Ic. Continuation of curve Ib. $pK_3' = 10.90$. IIa. 0.0278 *M* lysine monohydrochloride vs. 0.1000 *N* HCl (in 20 per cent dioxane). $pK_1' = 2.20$. IIb. 0.0278 *M* lysine monohydrochloride vs. 0.1000 *N* NaOH (in 20 per cent dioxane). $pK_2' = 9.00$. IIc. Continuation of curve IIb. $pK_3' = 10.70$.

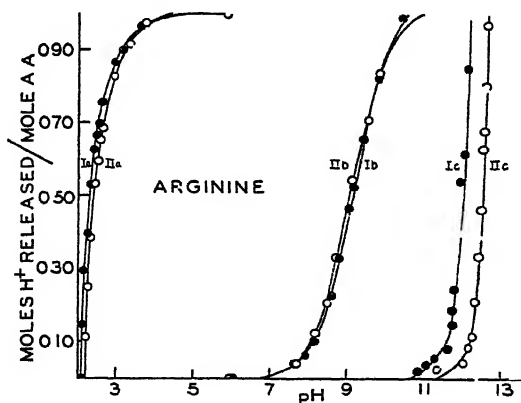


FIG. 8. TITRATION CURVES OF ARGinine MONOHYDROCHLORIDE IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0300 *M* arginine monohydrochloride vs. 0.1000 *N* HCl (in water). $pK_1' = 2.31$. Ib. 0.0300 *M* arginine monohydrochloride vs. 0.1000 *N* NaOH (in water). $pK_2' = 9.14$. Ic. Continuation of curve Ib. $pK_3' = 11.95$. IIa. 0.0239 *M* arginine monohydrochloride vs. 0.1024 *N* HCl (in 20 per cent dioxane). $pK_1' = 2.42$. IIb. 0.0239 *M* arginine monohydrochloride vs. 0.1000 *N* NaOH (in 20 per cent dioxane). $pK_2' = 9.04$. IIc. Continuation of curve IIb. $pK_3' = 12.50$.

Note: See Fig. 1, curves IV and V with reference to shift in pK_3' .

DISCUSSION

(a) *Acetic Acid*. The pK' value for acetic acid in 20 per cent dioxane is somewhat greater than in aqueous solution. It would not be expected that the pK' value for the more dilute solution would check with the thermodynamic constant reported by Harned and Kazanjian (8, 21) since the latter value was obtained by the use of cells without liquid junction and by extrapolation to zero ionic strength.

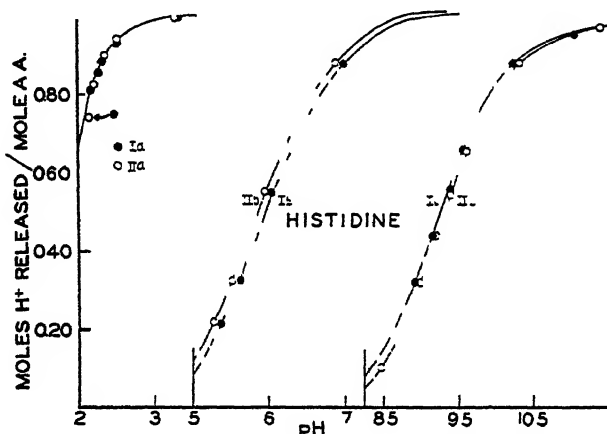


FIG. 9. TITRATION CURVES OF HISTIDINE DIHYDROCHLORIDE IN WATER AND IN 20 PER CENT DIOXANE

Ia. 0.0100 *M* histidine dihydrochloride vs. 0.1057 *N* NaOH (in water). $pK_1' = 1.90$. Ib. Continuation of curve Ia. $pK_2' = 5.95$. Ic. Continuation of curve Ib. $pK_3' = 8.75$. IIa. 0.0100 *M* histidine dihydrochloride vs. 0.1057 *N* NaOH (in 20 per cent dioxane). $pK_1' = 1.85$ – 1.90 . IIb. Continuation of curve IIa. $pK_2' = 5.55$. IIc. Continuation of curve IIb. $pK_3' = 8.81$.

(b) *α -Alanine*. The pK' values for aqueous solutions of α -alanine are a little larger than the thermodynamic values reported by Nims and Smith (22). The pK_1' value is somewhat larger when the amino acid is dissolved in 20 per cent dioxane than in water while the pK_2' value is practically unaffected.

(c) *β -Alanine*. The pK' values for the aqueous solution agree with those reported by Kirk and Schmidt (23). The effect of dioxane is to increase the pK_1' values. The pK_2' values in both solvents are nearly the same.

(d) *γ-Amino-n-valeric Acid*. For aqueous solution, the values are:

$$pK'_1 = 4.13$$

and

$$pK'_2 = 10.43.$$

The corresponding values given by Kirk and Schmidt (23) are 4.03 and 10.43. The pK'_1 value is increased in 20 per cent dioxane, while the pK'_2 value in dioxane-water is a little less than in water.

(e) *δ-Amino-n-valeric Acid*. The pK' values for the aqueous solution agree within 0.02 unit with those reported by Kirk and Schmidt (23). In 20 per cent dioxane, the pK'_1 value is increased while the pK'_2 value is decreased by a smaller amount.

(f) *Glutamic Acid*. In general the pK' values for the aqueous solution agree with those reported previously except that the pK'_3 value obtained is 9.88 instead of 9.66 (23). All pK' values are increased when this amino acid is dissolved in 20 per cent dioxane.

(g) *Lysine*. The pK'_1 and pK'_2 values for the aqueous solution check with the corresponding values given by Kirk and Schmidt (23) while the pK'_3 value (10.90) is several pH units more alkaline than the pK'_3 value reported by them. The effect of dioxane is to increase the pK'_1 value and to decrease the pK'_2 and pK'_3 values slightly.

(h) *Arginine*. The apparent dissociation constants for the aqueous solutions are:

$$pK'_1 = 2.31;$$

$$pK'_2 = 9.14;$$

$$pK'_3 = 11.95.$$

The corresponding values given by Kirk and Schmidt (23) are: 2.04, 9.04, and 12.48. The discrepancy in the pK'_1 value is due to the fact that curves Ia and IIa in Fig. 8 were plotted without applying the corrections for the titration of the solvent. When this correction is made, the pK'_1 value for the aqueous solution is 2.10 and that for the 20 per cent dioxane solution is 2.20. The value of pK'_1 is increased slightly in 20 per cent dioxane while the pK'_2 value is decreased. The pK'_3 value is probably not influenced to any extent. No definite statement regarding the change in pK'_3 can be made. This statement also applies to guanidine.

(i) *Histidine*. The pK' values for the aqueous solution check fairly well with those reported by Schmidt and coworkers (24). The pK'_1

value in 20 per cent dioxane is essentially the same as that in water while the pK'_2 value is decreased slightly and that of pK'_3 is increased by a smaller amount.

It is of interest to note the change in pK' values from those in water of certain amino acids and related compounds when they are dissolved in solvents of lower dielectric constants than that of water. These data are given in Table I.

In the following discussion all ionization constants are considered as acid constants in accordance with Bronsted's (25) definition. The ionization of weak acids in a particular solvent-water mixture depends on the activity of water in the mixture, the pK_a value, which becomes very important in alkaline solution, and the dielectric constant of the medium. In addition, such factors as the sizes of the ions and of the solvent molecules, the amount of hydration of the solvent, and the extent of association of the multipolar ions also play a rôle although probably to less an extent.

The data given in Table I show that, in the case of the monoamino-monocarboxylic acids, all of the $\Delta pK'_1$ values for all of the media are positive. The decreased dissociation of the carboxyl groups with decrease in the dielectric constant of the medium is probably not directly related to the decrease in dielectric constant since there is no neutralization of charges or disappearance of ions. In the case of acetic acid the pK value may be correlated with the water activity of the medium. Thus, Harned (10), and Harned and Fallon (11) have shown that there is a linear relationship between pK for acetic acid in a dioxane-water medium and the mole fraction of dioxane present. This indicates an inverse proportionality between the pK shown by an acid in a medium and the water activity of the medium. This is probably also true for the amino acids. The mole fraction of water present in a medium is an approximate substitute for a_{H_2O} . The proportionality between the water present in the solvent mixture and the quantity of "free" protons furnished by any acid is a direct result of the requirement that the proton must be hydrated (or solvated) in order to be dissociated.

MacInnes (26) and Greenstein (27) have considered the effect of position of the amino group in homologous amino acids on the dissociation constants in water. The relationships which they have postulated do not involve an assumption as to the actual mechanism of induction between the substituent and the dissociating group. They consider that the important factor for the magnitude of an inductive effect is the distance

TABLE I

The Changes in pK' Values of Certain Amino Acids and Related Compounds in 20 per cent Dioxane, 65 per cent Ethanol (2), and 86 per cent Ethanol (30)^a

Compound	20 per cent dioxane		65 per cent ethanol		86 per cent ethanol	
	$\Delta pK'$ of -COOH group	$\Delta pK'$ of -NH ₂ or other basic group	$\Delta pK'$ of -COOH group	$\Delta pK'$ of -NH ₂ or other basic group	$\Delta pK'$ of -COOH group	$\Delta pK'$ of -NH ₂ or other basic group
α -Alanine	0 23 ¹	-0 05 ¹	1 19	0 30	1 79 ⁷	0 13
β -Alanine	0 27 ¹	-0 07 ²				
γ -Amino- <i>n</i> -valeric acid	0 23 ¹	-0 07 ²	1 33	0	1 60 ⁸	
δ -Amino- <i>n</i> -valeric acid	0 30 ¹	-0 23 ²	1 53	-0 22		
ϵ -Aminocaproic acid					2 13 ⁸	
Glutamic acid	0 25 ¹	0 25 ²	0 97	1 09	1 65	0 15
	0 33 ²		1 38		1 42	
Lysine	0 10 ¹	-0 15 ²	1 05	0 05	1 73	0 18
		-0 20 ²		0		0 14
Arginine	0 11 ¹	-0 10 ²	1 32	0 36	1 12	-0 01
		0 55 ²		1 52		0 25
Histidine	0 ¹	-0 10 ²		-0 15		0 11
		0 06 ²	1 18	0 28	1 12	0 02
Acetic Acid	0 37		1 71		2 24 ⁸	
Monochloro-acetic Acid			1 58			
Lactic Acid			1 59 ⁴		2 25 ⁴	
Ethylamine				-0 62		-0 87
Cadaverine				-0 35 ¹		
				-0 66 ²		
Guanidine Sulfate		0 58				

¹ Values so labelled in this and corresponding columns are referred to in the text as pK'_1 .

² Values so labelled in this and corresponding columns are referred to in the text as pK'_2 .

³ Values so labelled in this and corresponding columns are referred to in the text as pK'_3 .

⁴ These values are taken from Michaelis and Mitzutani (3) and were obtained at 20°. Their value for the pK' of lactic acid in aqueous solution is 3.71. This is about 0.15 unit less than the thermodynamic pK found by Nims and Smith (22).

⁵ A value of 2.39 may be calculated from the data reported by Michaelis and Mitzutani (3) for the determination of the pK' of acetic acid in the medium at 19°.

⁶ Values so marked are taken from Edsall and Blanchard (4). These pK' values were determined in cells with liquid junctions at 25°.

⁷ Edsall and Blanchard (4) reported a pK' value for alanine in 86 per cent ethanol which yields a pK'_1 value of 1.46.

⁸ The solvent mixtures are in terms of weight per cent of the non-aqueous solvent. The following values appertain to the data in the table. (a) 20 per cent dioxane, dielectric constant = 60.8 (H_2O = 78.54); mole fraction of water = 0.533; pK_w = 14.62 (H_2O = 13.99); (b) 65 per cent ethanol, dielectric constant = 40; mole fraction of water = 0.560; pK_w = 15.5 (35); (c) 86 per cent ethanol, dielectric constant = 30; mole fraction of water = 0.300; pK_w = 16.6 (35). Unless otherwise stated, these values are taken chiefly from Robinson and Harned (12).

between the charge or dipole of the substituent group and the dissociating proton. The idea that the inductive effects of dipoles or of charged groups are exerted directly upon the acidic proton was expressed previously by Schwarzenbach and Egli (28) and by Neuberger (29). The latter appears to be the first to apply the concept that the inductive effect (which he terms the charge effect) is the "influence of the charge conducted through the dielectricum of the solvent." In the present discussion the assumption is made that, in the case of the monoamino-monocarboxylic acids where the substituent is the $-\text{NH}_3^+$ group, as the dielectric constant is lowered that portion of the inductive effect which is transmitted directly through the medium exerts increased force upon the proton of the carboxyl group.

Positively charged substituents, such as the $-\text{NH}_3^+$ group, will have an increased acid-strengthening effect as the dielectric constant of the medium is lowered. This will oppose that portion of the medium effect of the water-solvent mixtures which is due to decrease in water activity. Thus, dissociation of acids stronger than water is decreased by transfer from aqueous solution to a medium with lowered water activity regardless of the change of dielectric constant. Change in dielectric constant will either aid or oppose the water activity effect. In the case of $\text{NH}_3^+ \cdot \text{R} \cdot \text{COOH}$, lowering the dielectric constant of the medium will oppose the water activity effect and the $\Delta pK'_1$ of the amino acid (hydrochloride) will be smaller than the $\Delta pK'$ for acetic acid. From consideration of all the data given in Table I, it is probable that the changes in inductive effect appear much more rapidly than do changes in the effects of independent ions on one another. This appears reasonable in view of the extremely short distances that separate the substituents from the dissociating group. The short intramolecular distances introduce difficulties with regard to the physical interpretation of this assumption regarding inductive effects. These difficulties will be discussed later.

The $\Delta pK'_1$ values for the monoamino-monocarboxylic acids differ in amount from the $\Delta pK'$ values for acetic acid in the same media. This difference represents the effect of the $-\text{NH}_3^+$ group by induction through the chain or directly through the distance between the $-\text{NH}_3^+$ and the dissociating proton.

The magnitude of this inductive effect decreases with distance both when the amino acid is dissolved in water and in the water-solvent mixtures. The $\Delta pK'_1$ values increase as the distance of the amino group from the dissociating group increases. The $\Delta pK'_1$ for ϵ -aminocaproic

acid in 86 per cent ethanol approaches the pK_1 for acetic acid in the same medium. That the two polar groups of this amino acid do not affect each other greatly by induction is shown by the fact that the pK' values in water (4.43 and 10.75) (4) approach the corresponding pK value for acetic acid (4.76) and the pK' value for ethylamine (10.7).

The variations in the pK'_2 values are concerned with the effect of the medium upon the inductive effect between the $-\text{COO}^-$ group and the dissociating $-\text{NH}_3^+$ group. Since the substituted amines are weaker acids than water, as the mole fraction of water decreases in the media, the pK_a value for the solvent mixture is increased. As this value increases, a given amount of added hydroxide will remove more and more hydrogen ions from the weak acid. The $\Delta pK'_2$ values represent variations in dissociation of the $-\text{NH}_3^+$ group in comparison to the change in dissociation of water (ΔpK_a) in the same medium. It is apparent from the changes in dissociation of ethylamine and cadaverine that unsubstituted amines or diamines exhibit approximately the same intrinsic strength in the media as in aqueous solution.

The lowered dielectric constant which accompanies the increased pK_a of the water-solvent media will tend to make the $\Delta pK'_2$ values for a given amino acid more positive since the acid-weakening induction (direct inductive effect) from the $-\text{COO}^-$ group will be more effectively transmitted to the protons of the $-\text{NH}_3^+$ group. The pK'_2 values of the monoamino-monocarboxylic acids change from a positive to a negative value as the distance between the polar groups increases. This is consistent with the idea that the $-\text{NH}_3^+$ group behaves much like an aliphatic amine when the carboxyl group is far removed along the hydrocarbon chain. The $\Delta pK'_2$ values for cadaverine in 65 per cent ethanol appear consistent with the concept of increasing effects as the dielectric constant of the solvent is lowered. It is to be expected that the $\Delta pK'_2$ values for lysine will reflect the changes in induction of all three groups upon one another.

In the case of glutamic acid, the $\Delta pK'_1$ value of the carboxyl group closest to the $-\text{NH}_3^+$ group is of the order of that of alanine when both are compared in 20 per cent dioxane solution. The $\Delta pK'_1$ of glutamic acid is likewise of the same order as those of alanine and glycine in 65 per cent ethanol. The $\Delta pK'_1$ value for glutamic acid in 86 per cent ethanol taken from the work of Neuberger (30) is a little greater than the values of $\Delta pK'_1$ reported by Edsall and Blanchard (4) for glycine and alanine. The values of Neuberger assume a better relative position to

each other in this medium if they are considered in relation to the higher $\Delta pK'_1$ that he reports for alanine.

The $\Delta pK'_2$ value for glutamic acid (distal carboxyl group) in 20 per cent dioxane approaches the $\Delta pK'$ value for acetic acid in this medium. The $\Delta pK'_2$ values in the ethanol-water media are considerably less than the pK'_1 values for acetic acid in the same media. The pK'_2 value for glutamic acid represents the summation of the acid-strengthening induction from the $-\text{NH}_3^+$ group and the acid-weakening induction from the $-\text{COO}^-$ group, both groups being approximately equidistant from the dissociating proton. $\Delta pK'_2$ represents the changes in these direct inductive effects with change in solvent. Decreasing dielectric constant of the media should have a large weakening effect upon the dissociation which results in the formation of ions with 2 negative charges. The value for $\Delta pK'_2$ in 65 per cent ethanol differs markedly from the $\Delta pK'$ values of the aliphatic amines. The $\Delta pK'_3$ value for glutamic acid in 86 per cent ethanol is out of line with the trend of like quantities in the other water-solvent media. It is possible, but not proven, that the glutamic acid ions are markedly associated in 86 per cent ethanol.

The $\Delta pK'_1$ values ($-\text{COOH}$ group) for lysine increase from 0.10 (20 per cent dioxane) to 1.73 (86 per cent ethanol). In each of the water-solvent media the values differ from those of acetic acid. This may be attributed to changes in the direct inductive effects from the other groups in the compound. This discussion is not concerned with the transmission of an inductive effect through polarization of the atoms and bonds of the chain. This transmission should not be affected by the properties of the medium. However, the inductive effect *as a whole* must be dependent upon medium properties, since the energy possessed by a group by virtue of its charge (or dipole) may be dissipated by orientation effects when the medium contains water or other highly polarizable molecules. The negative $\Delta pK'_2$ and $\Delta pK'_3$ values for lysine possess an order of magnitude that are intermediate between the $\Delta pK'_2$ values of the α - and β -amino acids and the value for $\Delta pK'_2$ of γ -amino-*n*-valeric acid. In the other media the values for $\Delta pK'_2$ and $\Delta pK'_3$ become increasingly positive. These changes are consistent with the idea that the $-\text{COO}^-$ group exhibits an increasingly acid-weakening tendency as the dielectric constant is lowered.

In order that an amine may exhibit a positive $\Delta pK'$ value, it must become a weaker acid than water in the same medium. The rather large magnitude of the effects of acid weakening groups upon the dissociation

of the $R-NH_3^+$ acids is better shown by a comparison of the $\Delta pK'_2$ values with the $\Delta pK'$ value for ethylamine in the same medium. The negative values for the amines are due to increase in pK_a with decrease in mole fraction of water. When the $\Delta pK'$ values become positive, it is probable that the increased effectiveness in the direct inductive effect of the substituents due to lowered dielectric constant of the medium has become predominant.

In the case of arginine, the changes in the value of pK' are due to changes in induction between the positively charged guanidino and $-NH_3^+$ groups and the proton of the carboxyl group. The $\Delta pK'_1$ value (1.12) in 86 per cent ethanol based on the data of Neuberger (30) should probably be a little greater than 2.0. Too great a blank correction was probably applied by Neuberger. When all of the $\Delta pK'$ values for the three basic amino acids are considered, the latter value appears quite probable. The -0.01 value for $\Delta pK'_2$ in 86 per cent ethanol (change in dissociation of the $-NH_3^+$ group) is not consistent with the concept that there is an increase in the effectiveness of direct induction as the dielectric constant is lowered. No explanation for the low value is at present evident. The $\Delta pK'_3$ value (change in dissociation of the guanidino group) is smaller than might be expected. It is possible but not proven, that the second and third dissociations of arginine are affected by association of the ampholyte (dipolar ions) in 86 per cent ethanol. Jukes and Branch (31) attributed the high basic strength of the guanidino group to resonance, favoring the guanidinium ion form. The $\Delta pK'_3$ values for arginine and guanidine in 20 per cent dioxane are in line with this idea.

From the close proximity of the imidazole group of histidine to the amino and carboxyl groups, it is to be expected that a decreased dielectric constant will greatly increase the repulsions between both positive substituents and the dissociating proton. The $\Delta pK'_1$ values in the three media do not begin to approach the $\Delta pK'$ values for acetic acid. Since the $\Delta pK'$ values for acetic acid are due to the decreases in the activity of water, the difference between the $\Delta pK'_1$ value for histidine and the $\Delta pK'$ value for acetic acid in the *same media* is due to the change in the direct inductive effect of the positively charged imidazole and the $-NH_3^+$ group upon the proton of the carboxyl group. The $\Delta pK'_2$ values (changes in the dissociation of the imidazole group) in 20 per cent dioxane and in 65 per cent ethanol are essentially the same. Both are negative. In 86 per cent ethanol the value becomes positive. According to Hill and Branch (32) the weakly acidic nature of the imidazole group of histidine is due to

resonance favoring the uncharged form. The group is more acidic than water. The pK' value should vary with the α_{H_2O} of the medium and with the change in inductive effects of the two other dissociating groups in close proximity. On the basis of the $\Delta pK'_2$ values, it appears that the direct inductive effect from the $-\text{NH}_3^+$ group and the chain inductive effect from the $-\text{COO}^-$ group are sufficiently large to account for the negative values in 20 per cent dioxane and in 65 per cent ethanol. These two inductive effects are opposed by the acid-weakening direct inductive effect of the $-\text{COO}^-$ group and the decrease in water activity of the two media. The positive $\Delta pK'_2$ value in 86 per cent ethanol is probably due to the predominance of the water activity effect over the two acid-strengthening effects previously mentioned. The $\Delta pK'_2$ values in the 3 media do not show any particular consistency. No ready explanation for this is evident.

There are certain difficulties in visualizing the effect of a lowered macroscopic dielectric constant upon the intramolecular inductive effects. In order that the dielectric constant of the medium can affect induction, there must be a statistical distribution of the non-electrolyte molecules between the two polar groups concerned. In the case of the α -amino acids this is an impossibility since the distance between the $-\text{NH}_3^+$ group and the closest oxygen atom of the carboxyl group is of the order of one Ångström unit. A statistical distribution of non-electrolyte molecules between the polar groups of long chain amino acids in which the amino and carboxyl groups are widely separated is not possible unless there is more free rotation of the carbon-carbon bonds than is now believed to exist.

Kirkwood and Westheimer (33) have attempted to correlate dissociation constants with structure by modifying the electrostatic theory of group interaction proposed by Bjerrum (34). They suggest the term "effective dielectric constant" (denoted as D_E) which determines the effectiveness of group interaction in the molecule. For short chain amino acids D_E is a function of (a) the internal dielectric constant, D_I , (b) the distance of the interacting protons from the center of the sphere, and (c) the radius of the sphere. The smaller amino acids such as glycine and alanine are assumed to be approximately spherical in shape while the longer chain amino acids approximate the shape of ellipsoids which differ in eccentricity. For the longer chain amino acids, D_E is a function of D_I and the degree of eccentricity of the ellipsoid. The term D_E appears to be used as an empirical correction term. Its use implies

that deviations of inductive effects from the theoretically predicted effects are due to changes in D_E . From a consideration of the factors which determine the value of D_E , its value should apply to the inductive effects within the molecule of a compound without regard to the properties of the medium used as solvent.

The suggestion is advanced that one of the most important factors that determine the value of D_E is the degree of interaction of the charged groups with the solvent. This interaction may be termed "hydration." The term is used to designate any orientation of water molecules through hydrogen bonding or dipole-dipole interaction. The orientation is presumably easily reversible and does not greatly change the properties of the water molecules so oriented. The data given in the present paper indicate that the degree of hydration of the substituent groups varies with the mole fraction of water present in the water-solvent media. The magnitude of the dissociation constants of acetic, lactic, and monochloroacetic acid in the two alcohol-water mixtures indicates that the degree of hydration, which may be small for acids with *uncharged* substituents, does not differ in the two media and that the strength of these acids is due to the inductive effect of the electro-negative groups.

The aliphatic amino acids probably possess a low "internal" dielectric constant. The chief component of the quantity D_E is then the degree of orientation of the solvent molecules about the charged group or groups. This concept considers that the dipolar ions behave like other ions in that the electrostatic field of the polar portions extends in all directions. That portion of the electrostatic field of a polar group which exerts its effect outwardly from the molecule will cause the formation of clusters of water molecules about the group, each water molecule being oriented according to the charge of the group. The energy possessed by a group by virtue of its charge may be dissipated either externally in an amount that determines the degree of orientation of the solvent or it may be dissipated through an inductive effect upon other groups in the same molecule. The mode of dissipation of the energy of the charged substituent will then vary with the distance between it and other polar groups on the same molecule. The amount of energy expended internally and externally will also depend on steric effects since the strongest orientation of the dipoles, or the strongest hydrogen bonds, occur within a very short distance of the charged groups of the solute or its bonding protons. The amount of solvent orientation should depend upon the temperature and the relative tendency possessed by the non-electrolyte molecules, if pres-

ent, for the formation of competitive hydrogen bonds with the water molecules.

SUMMARY

1. The apparent dissociation constants of a number of amino acids and related compounds have been determined in water and in 20 per cent dioxane-water mixtures.

2. Comparisons have been made between the apparent dissociation constants in water, 20 per cent dioxane, 65 per cent, and 86 per cent ethanol.

3. The pK' values in the solvent mixtures appear to depend upon (a) the activity of water in the particular medium; (b) the ionization constant of water in the solvent mixture. This factor is of particular importance in the case of acids weaker than water; (c) the dielectric constant of the solvent; (d) variations in the inductive effect of polar groups (or dipoles) upon the proton of the dissociating group of the molecule with change in the water content of the medium. The increase in induction appears to accompany the decrease in dielectric constant of the medium but is more likely related to the water content of the medium.

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The Effect of Neutral Salts on the Dissociation of Certain Amino Acids in Dioxane-Water Mixtures¹

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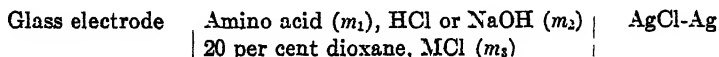
In a series of papers Batchelder and Schmidt (1-3) have presented studies on the effect of neutral salts on the dissociation of amino acids. For purposes of electromotive force measurements cells with and without liquid junctions were used. They showed that the effects of salts on the ionization of amino acids are the same as on the ionization or activity coefficients of other electrolytes. At low salt concentrations, the form of the curves showing the changes in the values of the apparent dissociation constants could be predicted on the basis of the theory of interionic attraction. The chief factors that determine the shape of the curves are the activity coefficients of the other ionic species that are involved in the ionization equilibrium. They found it necessary to use a "salting out" constant to obtain agreement between the experimental and the calculated values at higher salt concentrations. They pointed out, since in solvents of low dielectric the effects of the electrostatic forces are increased and those of the non-electrostatic forces tend to disappear, that the relative importance of these forces in determining the effects of salts on the ionization of amino acids could be estimated by carrying out experiments in solvents of lower dielectric constants than that of water.

EXPERIMENTAL

The present experiments were undertaken with this purpose in mind. However, certain experimental difficulties make it impossible to evaluate the data quantitatively. The data are, therefore, to be considered as semi-quantitative only.

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The solvent used was 20 per cent dioxane by weight. The mole fraction of water in the solvent mixture was 0.95. The concentration of added salt in the most concentrated solutions was low, the ionic strength being less than 0.16. E. M. F. measurements were made with the aid of the glass electrode in the manner previously described (4), using a saturated KCl bridge and, in certain instances, using a cell of the type



where m = molality of the constituents as indicated. The Ag-AgCl electrodes were prepared according to Brown (5).

The E. M. F. measurements, when the above cell was used, were evaluated in term of pH as follows:

$$E = E_0 - \frac{RT}{nF} \ln \alpha_{\text{H}^+} \alpha_{\text{Cl}^-} \quad (1)$$

$$E = E_0 - \frac{RT}{nF} \ln m_{\text{H}^+} m_{\text{Cl}^-} - nF \ln \gamma_{\text{H}^+} \gamma_{\text{Cl}^-} \quad (2)$$

$$-\log m_{\text{H}^+} = \frac{(E - E_0)nF}{2.303 RT} + \log m_{\text{Cl}^-} + 2 \log \gamma_{\text{HCl}} \quad (3)$$

where E = measured E.M.F. of the cell, E_0 = standard E.M.F. of the cell, and T = absolute temperature. The constants R , n , and F have their usual values. α = activity of the constituent denoted by the subscript. The activity terms have been replaced by the equivalent terms. The pH was taken equal to $-\log m_{\text{H}^+} d_0$ where d_0 = the density of the 20 per cent dioxane. E_0 was estimated by measuring the potential of the AgCl-Ag cell using solutions of HCl in 20 per cent dioxane. The E values for several molalities of HCl were compared with the corresponding E values obtained by Harned and Morrison (6). In estimating E_0 the assumption was made that the glass electrode itself contributed a constant potential. The potential of the glass electrode is constant for a small pH range (2-3 units). The E_0 value, when the glass electrode is immersed in acidic solutions (pH 2-4), will not be the same as when strongly alkaline solutions are used. This is due to a change in the "asymmetry potential" of the glass and is difficult to estimate. The following values for E_0 were taken: acidic region, -0.4914 volt; alkaline region, -0.4895 volt.

The difficulty in using cells without liquid junctions when solvent mixtures containing salts are used is due to lack of data for γ_{HCl} . The values for $-\log m_{\text{H}^+}$ in each of the solutions determined were calculated with the aid of equation (3) using values of $-\log \gamma_{\text{HCl}}$ in the following way. A large scale plot was made of the values of $\log \gamma_{\text{HCl}}$ vs. m_{HCl} taken from the work of Harned and Donelson (7). The assumption was made that γ_{HCl} in solutions of amino acid, HCl (or NaOH), and MCl is the same as in solutions of HCl of the same ionic strength. The plotted curve was used as if it were a plot of $\log \gamma_{\text{HCl}}$ vs. μ . The other values in equation (3) are known or may be calculated.

Due to the presence of neutral salts, the uncertainty of the value of the liquid junction potential arises when cells with the liquid junction potential are used. To a certain extent this varies with the pH of the solution under test. The liquid junction potential is greatly magnified at pH values where it is necessary to apply a correction for free hydrogen or hydroxyl ion. The titration of solvent alone follows an hyperbolic curve. A slight error in estimating the blank correction for the dilute amino acid solutions will result in a large change in the ratio that determines pH.

Several experiments were carried out in which the pH of identical solutions was determined by the use of both types of cells. It was assumed that the difference between the values was due to the liquid junction potential, an assumption which undoubtedly is somewhat arbitrary. Only changes in potential greater than these differences were considered as being significant for purposes of interpreting the data. It should be borne in mind that the errors in estimating the last term of equation (3) are probably fully as large as those made by the use of cells with liquid junctions. The data obtained by means of the two types of cells are given in Table I.

The data obtained for the effect of neutral salts on the dissociation of certain amino acids are represented graphically in Figs. 1-17. Cells with liquid junctions of the type already described (4) were used in obtaining the data. The pH readings were calculated in terms of the apparent dissociation constants for the amino acid under consideration. The total ionic strength of each solution was calculated from the concentrations of all the ionic constituents and their valences. Although the zwitterion adds to the ionic strength of a solution, its contribution was neglected in the summations.

TABLE I

Comparison of pH Measurements in Cells (a) with and (b) without Liquid Junctions

m_2	pH cell (a)	pH cell (b)	Difference
I. Glutamic acid, $m_1 = 0.00967$; HCl, $m_2 = 0.00482$			
NaCl			
0.00984	2.79	2.79	0
0.0295	2.81	2.79	-0.02
0.0492	2.81	2.82	+0.01
0.0689	2.82	2.80	-0.02
0.196	2.80	2.78	-0.02
KCl			
0.00984	2.79	2.76	-0.03
0.0295	2.80	2.76	-0.04
0.0492	2.80	2.75	-0.05
0.0689	2.81	2.78	-0.03
0.196	2.82	2.76	-0.06
II. δ -Amino- <i>n</i> -valeric acid, $m_1 = 0.00988$; HCl, $m_2 = 0.00482$			
NaCl			
0.00984	4.62	4.66	+0.04
0.0689	4.65	4.66	+0.01
KCl			
0.00984	4.63	4.65	+0.02
0.0689	4.65	4.64	-0.01
LiCl			
0.00800	4.64	4.66	+0.02
0.0562	4.66	4.65	-0.01
III. δ -Amino- <i>n</i> -valeric acid, $m_1 = 0.00988$; NaOH, $m_2 = 0.00482$			
NaCl			
0.00985	10.54	10.53	-0.01
0.0197	10.51	10.49	-0.02
0.0492	10.50	10.48	-0.02
0.0689	10.49	10.47	-0.02
0.197	10.43	10.39	-0.04
KCl			
0.00985	10.55	10.50	-0.05
0.0197	10.51	10.47	-0.04
0.0492	10.50	10.45	-0.05
0.0689	10.50	10.43	-0.07
0.197	10.47	10.38	-0.09

Note: All materials were dissolved in 20 per cent by weight dioxane solution. The terms m_1 , etc. refer to molalities of constituents. Cell (a) contains a liquid junction between the solution under test and the aqueous saturated KCl solution. The Ag-AgCl electrode was used in cell (b). The glass electrode was used in

DISCUSSION

The curves for α -alanine (Fig. 1) possess considerable individuality for the reason that variations in liquid junction potential are large in acidic solutions and the need for correcting the pH for free hydrogen ions magnifies the differences in liquid junction potential. The average slope of the curves for the univalent chlorides is the same as that of the slope for the same salts when aqueous solutions were used. Due probably to the smaller liquid junction potential and lack of the necessity for the blank correction, the agreement among the effects of the univalent chlorides in alkaline solution (Fig. 2) is better than in acidic solution.

In the case of β -alanine (Fig. 3) the agreement among the univalent chlorides is better than in the case of α -alanine due probably to the

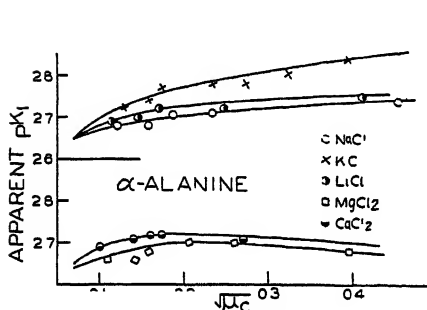


FIG. 1. The effect of salts upon pK'_1 of α -alanine in 20 per cent dioxane solution.

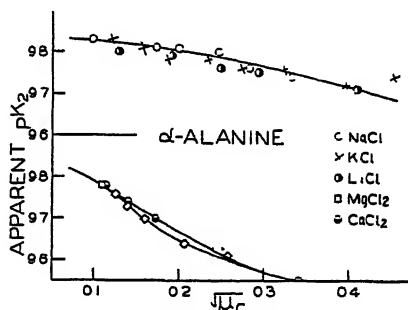


FIG. 2. The effect of salts upon pK'_2 of α -alanine in 20 per cent dioxane solution.

smaller variations in liquid junction potential over the pH region studied. The apparent pK'_2 of β -alanine (Fig. 4) is affected in more individual fashion by the presence of chlorides than in the case of α -alanine. The curve for lithium chloride stands out in this respect.

The effects of the monovalent chlorides upon the dissociation constants of γ -amino-*n*-valeric acid (Figs. 5 and 6) are qualitatively the same as in the case of β -alanine except that the effects of potassium chloride (Fig. 5) are more positive. There is no evident reason for this.

The monovalent chlorides affect the dissociation constants of δ -amino-*n*-valeric acid (Figs. 7 and 8) in much the same manner as in the case of the other amino acids already discussed. As in the case of β -alanine and γ -amino-*n*-valeric acid, the effects of lithium chloride are less positive on the pK'_2 of δ -amino-*n*-valeric acid than in the case of sodium and potassium chlorides.

The curves showing the effects of calcium and magnesium chloride on the pK'_1 of the four amino acids have a more negative slope than in the case of the univalent chlorides. There is no large difference between the effects of the two divalent chlorides. In alkaline solutions the curves for the two divalent chlorides differ considerably in pH regions

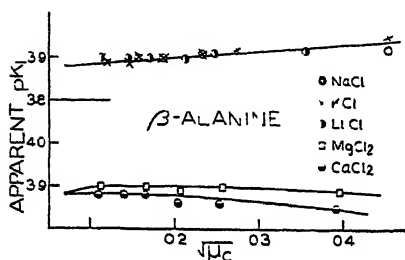


FIG. 3. The effect of salts upon pK'_1 of β -alanine in 20 per cent dioxane solution.

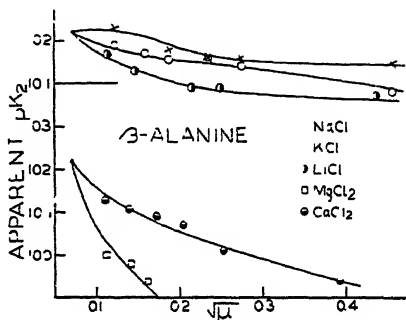


FIG. 4. The effect of salts upon pK'_2 of β -alanine in 20 per cent dioxane solution.

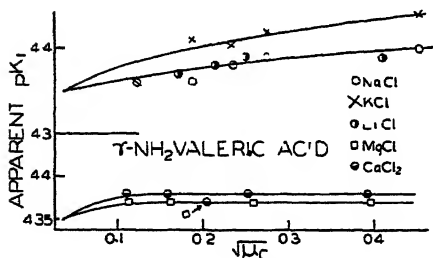


FIG. 5. The effect of salts upon pK'_1 of γ -amino-*n*-valeric acid in 20 per cent dioxane solution.

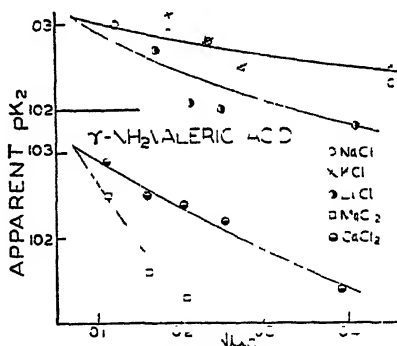


FIG. 6. The effect of salts upon pK'_2 of γ -amino-*n*-valeric acid in 20 per cent dioxane solution.

of 10.0 or more. The steep negative slope for magnesium chloride is due chiefly to the formation of insoluble magnesium hydroxide.

The curves for the effect of sodium, lithium, calcium, and magnesium chloride on the pK'_1 of *lysine* have similar slopes (Fig. 9). The slope of the potassium chloride curve is somewhat more positive. This was

also evident in the case of α -alanine and γ -amino-*n*-valeric acid. The effects of sodium and potassium chlorides on the pK'_2 of lysine (Fig. 10) are identical. The slope is much like that showing the effects of the several chlorides on the pK'_1 of lysine. It differs from the curves of the first four mentioned amino acids in that the slope is positive whereas the

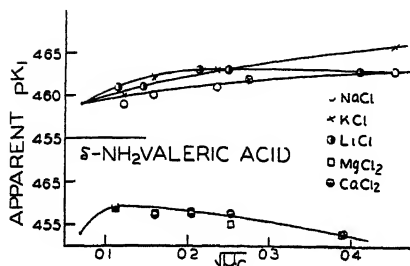


Fig. 7. The effect of salts upon pK'_1 of δ -amino-*n*-valeric acid in 20 per cent dioxane solution.

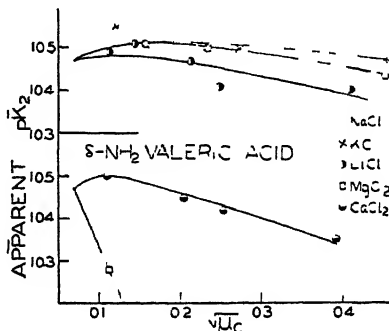


Fig. 8. The effect of salts upon pK'_2 of δ -amino-*n*-valeric acid in 20 per cent dioxane solution.

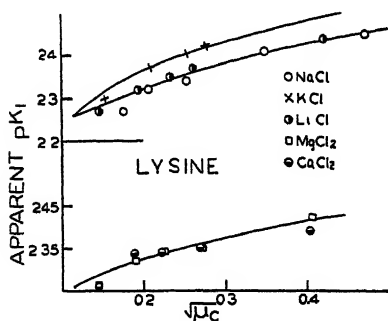


Fig. 9. The effect of salts upon pK'_1 of lysine in 20 per cent dioxane solution.

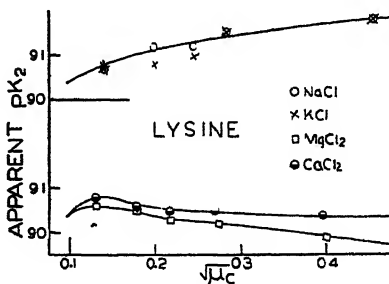


Fig. 10. The effect of salts upon pK'_2 of lysine in 20 per cent dioxane solution.

slopes were negative in the case of the four monoamino-monocarboxylic acids. This appears to be related to the second positively charged substituent of lysine. As in the case of δ -amino-*n*-valeric acid, the curves showing the effect of calcium and magnesium chloride upon the pK'_2 of lysine show maxima at low ionic strength. Thereafter the slope decreases. This type of curve is characteristic of the strong interactions

between the zwitterion of lysine ($-\text{COO}^-$ group) and the small divalent ions and is probably due to the formation of complex ions or compounds that are not very highly dissociated. The effects of sodium and potassium chlorides on the pK'_3 of lysine (Fig. 11) are essentially the same. The curve for lithium chloride is more negative and resembles the

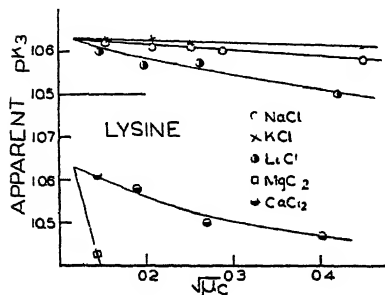


FIG. 11. The effect of salts upon pK'_3 of lysine in 20 per cent dioxane solution.

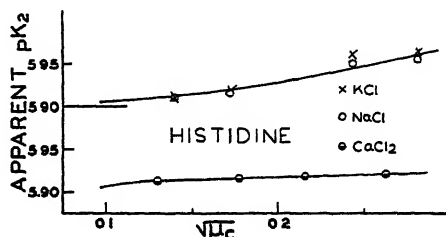


FIG. 12. The effect of salts upon pK'_2 of histidine in 20 per cent dioxane solution.

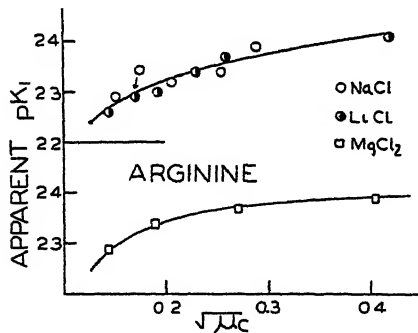


FIG. 13. The effect of salts upon pK'_1 of arginine in 20 per cent dioxane solution.

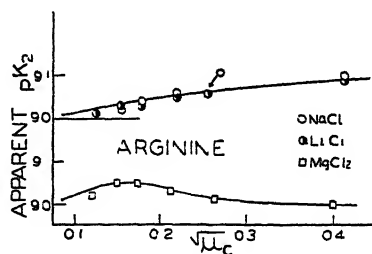


FIG. 14. The effect of salts upon pK'_2 of arginine in 20 per cent dioxane solution.

calcium chloride curve. This can perhaps be due to decrease in the activity of the corresponding hydroxides.

The effects of the varying concentrations of the three chlorides on pK'_2 of *histidine* (Fig. 12) are similar. The small difference between the curves showing the effect of calcium chloride upon the pK'_2 of lysine and of histidine may be correlated with the structures of the two amino acids.

In the case of lysine the —COO^- group possesses fairly independent behavior since the —NH_3^+ group is separated by five —CH_2 groups from the carboxyl group. The zwitterion of histidine presumably has its positive charge residing upon the α -amino group so that the fields of the —COO^- group and the —NH_3^+ group are largely "neutralized" by the proximity of the two groups to each other.

In the case of *arginine* the effects of sodium, lithium, and magnesium chloride upon pK'_1 (Fig. 13) are quite similar. The curves show very rapid changes of pK'_1 with very small change in ionic strength. This may, in part, be due to a rapid change in liquid junction potential. The curves shown in Fig. 14 resemble those for the pK'_2 of lysine. The mag-

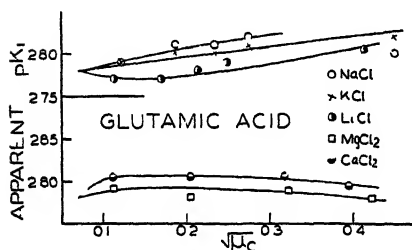


Fig. 15. The effect of salts upon pK'_1 of glutamic acid in 20 per cent dioxane solution.

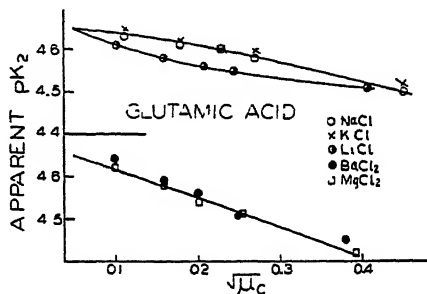


Fig. 16. The effect of salts upon pK'_2 of glutamic acid in 20 per cent dioxane solution.

nesium chloride curve shows a maximum at low ionic strength. This may, in part, be due to a rapid change in liquid junction potential. The curves shown in Fig. 14 resemble those for the pK'_2 of lysine. The magnesium chloride curve shows a maximum at low ionic strength. This is due to combination of the magnesium ion with the —COO^- group of the arginine zwitterion.

The effect of sodium and potassium chloride on the pK'_1 of *glutamic acid* (Fig. 15) is much the same as in the case of the other α -amino acids. The lithium chloride curve, however, is somewhat different. This is also evident in Fig. 16. However, the effect of lithium chloride on the pK'_2 of glutamic acid (Fig. 17) is essentially the same as that of potassium chloride and somewhat different from that of sodium chloride. It is not at all certain that the deviations in behavior found among the several univalent chlorides can be taken in an absolute sense. They may

fall within the category of the variables inherent in the experimental procedures. However, deviations among the several univalent chlorides were also found by Batchelder and Schmidt (2, 3). The effects of calcium and magnesium chloride on the pK'_1 of glutamic acid are similar. The curves show slight maxima at low ionic strengths. The identical curves (Fig. 15) showing the effects of barium and magnesium chlorides on the pK'_2 of glutamic acid are approximately parallel to the curves for the univalent chlorides (Fig. 16). The three divalent chlorides affect

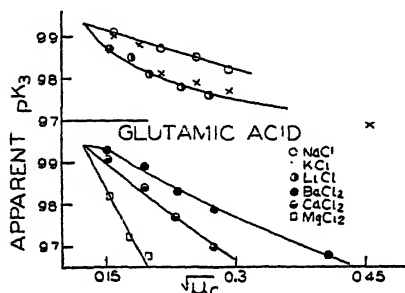


FIG. 17. The effect of salts upon pK'_2 of glutamic acid in 20 per cent dioxane solution.

the pK'_3 of glutamic acid quite differently. The steep curves for calcium and barium chloride indicate that the divalent ions form complexes or compounds which glutamic acid forms with univalent cations. The very steep slope of the magnesium chloride curve indicates formation of magnesium hydroxide as well as formation of complex ions with glutamic acid.

Batchelder and Schmidt (2) employed the following equation to describe the effect of chloride salts upon the first apparent dissociation constant of alanine:

$$-\log K'_1 = -\log K_1 + \log \gamma_{R^{+-}} + C\sqrt{\mu_c} + B\mu_c \quad (4)$$

where K'_1 is the apparent dissociation constant, K_1 is the thermodynamic dissociation constant, $\gamma_{R^{+-}}$ is the activity coefficient of the dipolar ion, μ_c is the ionic strength of the solution, C is an empirical constant, and B represents a "salting out" term. At low ionic strengths the empirical terms $C\sqrt{\mu_c}$ and $B\mu_c$ may be neglected and the difference between $-\log K'_1$ and $-\log K_1$ should be equal to $-\gamma_{R^{+-}}$. $-\log \gamma_{R^{+-}}$ may be estimated by Kirkwood's (8) equation. For very dilute aqueous solutions of the zwitterions and in a medium having a dielectric constant of 60.8 (20 per cent dioxane) and at 25°, Kirkwood's equation takes the form:

$$-\log \gamma_{R^{+-}} = 0.209 \frac{R^2 \mu_c}{a} \frac{1}{1 + 0.373a\sqrt{\mu_c} + 0.046a^2\mu_c + 0.023 \frac{b^3 \mu_c}{a}} \quad (5)$$

If the chief factors in determining the interactions between dipolar ions and the ions of the salt are electrostatic, a change in the macroscopic dielectric constant should result in a proportional change in equation (5) by which γ_{R+} is estimated. It should also result in a calculable change in $-\log \gamma_i$ of any of the ions present in the medium. If the only effect of the change of medium from water to 20 per cent dioxane is to change the dielectric constant from 78.5 to 60.8, there should be a demonstrable change in slope of the curves that represent the effects of the salts upon the dissociation constants of the amino acids. The change in slope will be a function of the dielectric constant only if the values assigned to a and b are identical with the values which must be assigned to the same salt-amino acid system in aqueous solution.

The results of the present study may be compared with the corresponding data in aqueous solution (1). The effects of sodium and magnesium chloride upon the pK'_1 of alanine are essentially the same in the two media. The slopes of comparable curves showing the effects of salts on the pK'_2 of alanine are quite similar in the two media. Like statements apply in those cases where the same amino acid and salt were used and when due consideration is given to the effect of the contact potential. These results are not wholly unexpected. Elliott and Kilpatrick (9) found that the determination of "comparable acid strengths" for substituted benzoic acids in dioxane-water mixtures did not yield the same results as those found for ethanol-water mixtures with equal dielectric constants. The term "comparable acid strengths" refers to determinations of the strength of each of the substituted acids relative to the strength of benzoic acid in the same medium.

Scatchard (10) has shown that there is a "sorting out" of solvent mixtures by ions. This sorting out apparently relates to the tendencies of the polar water molecules to orient their dipoles about the ions or polar groups of the solute molecules. From the theoretical treatment of solvent mixtures by Debye (11), Scatchard calculated, in the case of ethanol-water mixtures, that little non-electrolyte would be present within one Ångström unit of the ion even in the most concentrated ethanol solutions. At a distance of 3 Ångström units from the ion, the medium would have its average composition. He concludes that it will be necessary to take the effects of size and structure of the non-electrolyte into consideration in order to explain the effect of orientation in solvent mixtures.

The effects of neutral salts upon the dissociation constants of amino

acids in 20 per cent dioxane, except in such cases as when complex ions are formed or precipitation of the metallic hydroxide occurs, appear to be due to sorting out of this mixture rather than the direct effect of a lowered dielectric constant. In both water and in the dioxane-water mixture, water molecules orient themselves about the salt ions and the dipolar ions. In the case of small ions, especially those with more than one valence, the orientation of water molecules may be so definite that an envelope of water molecules travels with the salt ions. In such a case, at the moment of "collision", this shell of water may be tangent to the shell of water about the polar dissociating group so that the dielectric constant of the medium between ion and group is almost that of water. If the salt effect results from the interactions of the ion atmospheres about different polar groups of a compound, the dielectric constant concerned must be that of the portion of the medium through which the "effect" passes. In a medium which contains a high mole fraction of water, as in 20 per cent dioxane, the clusters of water molecules about the ions and dipolar ions are probably almost maximal so that it would seem probable that the portion of the medium through which the effect is transmitted is essentially aqueous in composition. Possibly one of the largest factors in determining the importance of "hydration" in water-solvent mixtures is the distance through which a given interaction or effect is transmitted. Thus, if effects are those transmitted through a short distance at the moment of collision of ion and molecule, then the degree of hydration of the solutes will be an important consideration, even in mixtures with low mole fractions of water.

Another effect must also be considered in evaluating the data. Addition of salt to water and to a solvent-water mixture will in both cases affect the dielectric constant of the media and, hence, the dielectric constant of a solution containing amino acid, salt, and solvent will be different from that of the solvent alone. The behavior of dipoles and of charged salt ions are alike to the extent that both will assume an orderly arrangement in an oscillating field. In the case of dipoles, an electric field is set up in a direction opposing the field set up when 2 charged condenser plates are immersed in such a medium and hence the potential difference between the plates is diminished. In other words, the capacity of the condenser is increased and this is reflected in an increase in the value of the dielectric constant of the solution. The analogy between the behavior of dipoles and simple ions ceases since the simple ions possess an actual, over-all charge and so are free to migrate

and to react at the electrodes. However, both the dipolar molecules and the salts are highly polar in comparison with the volume of water they displace so that both should be considered to possess "dielectric increments" for their aqueous solutions.

It appears, therefore, important to emphasize that the dielectric constant of the solvent or of a solvent mixture should not be considered a rigorous parameter of the system, but rather that it is a constant which remains unchanged only so long as there are no appreciable concentrations of polar substances or salts present.

In the work of Batchelder and Schmidt (2, 3) two empirical constants were utilized in the equation which described the conditions in the amino acid solutions containing salts. It may be possible that in part the empirical corrections are required because the dielectric constants of the systems have far outranged that of the solvent. It appears probable that the effect of the salts in the 20 per cent dioxane solutions, to a large extent, counteracts that due to the dioxane upon the dielectric constant of the medium. Thus, the almost identical effect of salt upon the dissociation of amino acids in water and in 20 per cent dioxane is in large part due to similar dielectric constants in the portions of the media through which the interactions between ions and polar groups are transmitted.

SUMMARY

1. The effects of certain neutral salts upon the apparent dissociation constants of α - and β -alanine, γ - and δ -amino-*n*-valeric acid, lysine, histidine, arginine, and glutamic acid dissolved in 20 per cent dioxane have been determined.

2. Comparison, when possible, have been made with previous work carried out in aqueous solution by Batchelder and Schmidt.

3. In comparable instances, the effect of the added salt upon the dissociation constants of amino acids was not materially different in the two solvents.

4. Possible explanations for this are given.

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The Effect of Dry Grinding on the Properties of Proteins

1. Native, Denatured and Coagulated Ovalbumin*

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Physical agents such as shaking, high pressure, formation of surface films and supersonic waves are capable of bringing about denaturation and coagulation of proteins. The number of different proteins studied up to this time have been too few to warrant including grinding as another important physical agent bringing about changes in the protein molecule which are associated with denaturation and coagulation. In this and subsequent papers it will be shown that the dry grinding of numerous proteins produces definite irreversible changes in the protein molecule.

Our attention was brought to this problem when it was found that collagen yielded some water soluble protein after having been ground in the ball mill.

Several investigators have shown that dry grinding has a definite effect on the proteins and polysaccharides they studied.

Kuhne (1) found that pepsin was able to digest hair keratin after it had been subjected to grinding. Alsberg and Perry (2) ground starch and found that it became soluble in cold water. Alsberg and Griffith (3) observed that gelatin became soluble in cold water after being ground in the ball mill. These authors ground wheat flour and found that the water insoluble colloids, gliadin and glutenin, were not rendered materially soluble by 16 hours grinding. Gluten prepared from excessively ground flour swells less than the control gluten. They concluded that grinding modifies the swelling property of gels by modifying the protein structure.

Boissevain (4) repeated the investigations of Alsberg and coworkers

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and found that the effect of grinding on starch was the same as the action of other hydrolytic agents. He also ground tubercle bacilli and obtained water soluble protein, peptone and polysaccharide. This author compares the effect of grinding to that of acid hydrolysis.

Routh and Lewis (5) compared the rates of digestion of wool ground for 75 and 175 hours with that of keratin produced by the reduction of wool with thioglycolic acid (6). The ground wool contained water soluble protein which gave a positive Biuret test, a slight precipitate with trichloroacetic acid and positive Folin and Marenzi and cyanide nitroprusside reactions. 36.6 per cent of the total nitrogen and 34.1 per cent of the sulfur were lost when the ground powder was dialyzed against running water.

Routh (7) carried out chemical studies on the effect of grinding on wool. The cystine and total nitrogen in the water soluble fraction increased with the time of grinding. The amino nitrogen varied but slightly and was practically constant and independent of the amount of grinding. Routh points out that "Boissevain's results indicate that the process is similar to acid hydrolysis. . . . Hydrolytic action alone cannot explain the results obtained in the present investigation. The mechanical process may produce a water soluble fraction merely by bringing the water soluble material into contact with the solvent. Whatever the process of degradation it is accompanied by oxidative changes."

It shall be our purpose in this and succeeding papers to show that dry grinding produces changes in the protein molecule which warrant its inclusion as another physical agent producing changes associated with denaturation and coagulation.

EXPERIMENTAL

The proteins studied and reported in this investigation were all ground under the following conditions. The dried proteins were placed in porcelain ball mills of one liter or eight liters capacity which were filled to half capacity with flint pebbles, the average diameter of which was $\frac{1}{8}$ ". The ball mill was rotated at approximately 100 revolutions per minute.

Native Ovalbumin. The ovalbumin used in this experiment was prepared by the method of Cole (8) and was recrystallized 4 times. Five grams of dry, crystallized ovalbumin were placed in a ball mill of one liter capacity and ground for 24 hours. The dry powder was removed from the mill and separated from the stones by passing through a 40 mesh sieve.

The ground powder was found to be mainly insoluble in water. The water soluble portion which was small in quantity gave a positive Biuret, was precipitated by half saturation with ammonium sulfate and was not coagulated by heating at the isoelectric point. The water insoluble residue contained an acid soluble fraction, soluble at pH 3.0 (HCl) and an alkali soluble fraction soluble at pH 9.5 (NaOH). The amount of protein dissolved in both cases was small and precipitated when the solutions were adjusted to the isoelectric point. These fractions have the solubilities of acid and alkali denatured proteins. The major portion of the ground ovalbumin was insoluble in water, dilute acid and alkali, and thus has a solubility similar to that of coagulated ovalbumin.

The residue insoluble in water, acid and alkali contained some protein soluble in one-half saturated urea solution. The remaining insoluble protein formed a highly opalescent solution of high viscosity in saturated urea solution, which after standing for a short time, became a gel.

TABLE I
Chemical Differences in Native Ovalbumin and Water Soluble Fractions

	Per cent Nitrogen	Per cent Sulfur	Per cent Tyrosine	Per cent Tryptophan
Preparation I	15.60	1.34	4.20	1.30
Preparation II	15.27	2.94	2.46	0
Preparation III	15.13	2.20	3.06	0.51

Coagulated ovalbumin required standing in saturated urea for 3-4 days before it disperses and forms a gel, never actually dissolving.

Acid Denatured Ovalbumin: The acid denatured ovalbumin was obtained by heating a dilute salt free solution of ovalbumin at pH 3 in a water bath at 100°C. for 20 minutes. The denatured protein was precipitated by adjusting the solution to the isoelectric point, pH 4.8, filtered on a Buchner funnel and spread on plates to dry. Five grams of dry denatured ovalbumin were ground in a mill of one liter capacity for a period of 36 hours. The ground powder was now almost entirely insoluble at pH 3.0, whereas the dry unground protein was approximately 50 per cent soluble at that pH. The acid soluble ovalbumin has been converted into an acid insoluble form.

Heat Coagulated Ovalbumin: The heat coagulated ovalbumin was prepared by heating a dilute solution of ovalbumin at its isoelectric pH and the coagulum was dried by passing a current of dried warm air over it. Thirty grams of dry coagulated ovalbumin were ground in a ball mill of eight liters capacity for a period of 48 hours. The powder was

extracted with distilled water until all of the water soluble protein was removed. The water soluble fraction was dried by evaporation at 38°C. in a current of dry air and represents Preparation II.

The insoluble residue was dried and again ground for 48 hours. The powder was extracted with water, dried and designated as Preparation III. An unground sample of dried crystallized ovalbumin constitutes Preparation I.

The nitrogen of the water soluble fractions was determined by the Kjeldahl method; the total sulfur gravimetrically as sulfate after oxidizing the organic matter: the tyrosine and tryptophan by the method of Folin and Marenzi (9). Table I shows the analytical results.

ELECTROPHORESIS STUDIES

Through the kind cooperation of Drs. D. A. MacInnes and L. G. Longsworth of Rockefeller Institute we were able to obtain an electrophoretic analysis of the water soluble fractions. All of the patterns were obtained with approximately 1 per cent solutions of the protein on a dry weight basis—in a buffer 0.025 molar in both Na_2HPO_4 and NaH_2PO_4 after electrolysis for 6000–6500 seconds at 7.3 volts per cm.

Included for comparisons are (a) a pattern of undried crystallized ovalbumin (supplied by Dr. MacInnes), (b) a pattern of our dried crystallized ovalbumin (Preparation I), (c) a pattern of heat denatured but not coagulated ovalbumin (Dr. MacInnes). Dr. MacInnes has carried out an electrophoretic analysis of a sample of dried ovalbumin submitted to him by Dr. A. G. Cole. The pattern (b) of our sample of dried ovalbumin corroborates the findings of Dr. MacInnes on Dr. Cole's sample that the dried ovalbumin is less homogeneous electrophoretically than the undried protein (Fig. 1).

The coagulated ovalbumin used in this experiment was completely insoluble in water and in dilute acids (pH 3.0), and alkali (pH 9.5). The ground powder has a light yellow color and a peculiar characteristic odor. The water soluble fractions produced by grinding are not coagulable by heat, are soluble in acids, in alkali and give a Biuret test (pink-violet) indicative of the lower protein degradation products. They are precipitated by half saturation with ammonium sulfate, trichloroacetic acid, phosphotungstic acid, and picric acid.

Preparations II and III contain high concentrations of dialyzable material, 89.77 per cent and 85.5 per cent respectively of the total nitrogen passing through Visking casing membranes upon complete dialysis.

After extraction of the water soluble protein from the ground coagulated ovalbumin, the residue contains fractions which are soluble at pH 3.0 (HCl) and pH 9.5 (NaOH).

As has been pointed out, patterns (a), (b), and (c) have been included for comparison. The water soluble protein from the second 48 hour period of grinding (Preparation III) dissolved or dispersed in the buffer solvent with very little material remaining in suspension that could be

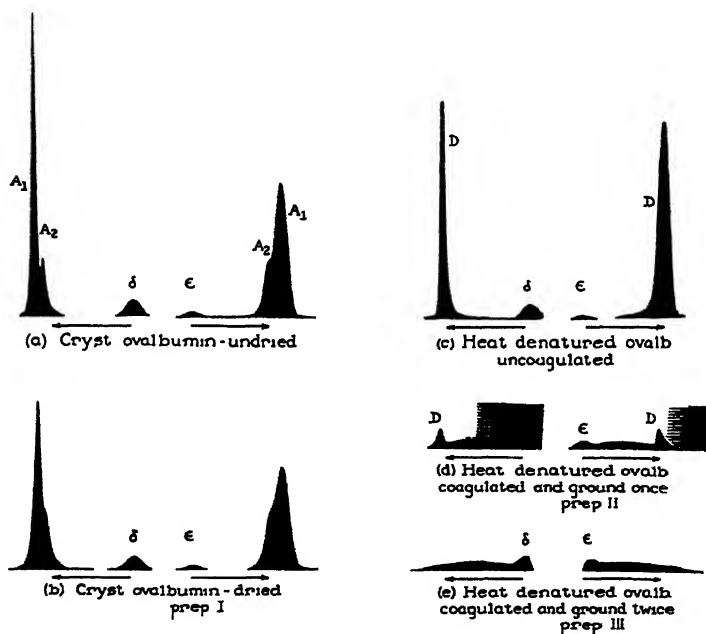


Fig. 1

Electrophoretic Analysis of Dried, Undried, Heat Denatured Ovalbumin and the Water Soluble Fractions Obtained by Grinding Heat Coagulated Ovalbumin

thrown out in the angle centrifuge, but the resulting solution had a pronounced opalescence. In distilled water the solutions are perfectly clear. The pattern (e) of this solution indicates fractions having all mobilities from zero to that of native ovalbumin. No one fraction appears to predominate. This behavior is in marked contrast to that of heat denatured but uncoagulated ovalbumin (c) in which the denatured protein is quite homogeneous electrically and has a mobility very similar to that of the native protein. A striking feature of the pattern

of the ground water soluble protein, however, is the relative absence of refracting material. Only about 45 per cent of the original material is accounted for by the total area under the refractive index gradient curve. This is due to loss of protein in the dialysis prior to electrophoresis, the grinding process having degraded the coagulated protein to a considerable extent.

The pattern (d), which represents Preparation II and is the water soluble protein split off during the first 48 hour period of grinding, shows some material (d) with the original mobility, the effect of grinding not having gone so far. It would appear that one fraction of the water

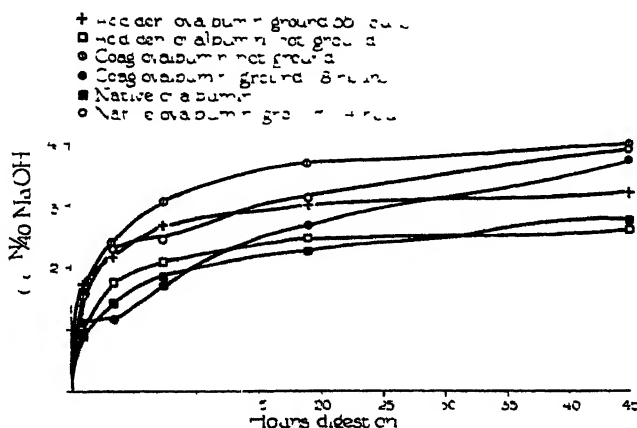


FIG. 2

Comparative Digestion Rates of Various Ovalbumins by Pepsin Rates
Determined by the Sorensen Titration Using $N/40$ NaOH

soluble protein of Preparation II has the mobility of the denatured ovalbumin.

Comparison of Rates of Digestion of Ground and Unground Ovalbumin: The digestion was carried out using Parke, Davis and Company pepsin (1-10,000). The concentration of protein in each digest was exactly the same and equal to 25 mg. of protein either dissolved or suspended in each cc. The suspensions were shaken at frequent intervals. The concentration of enzyme was 4.6 mg. of pepsin protein in each cc. The pH was 1.7 and the digestion was carried out at 37°C. The digestion was followed by the Sorensen titration (Fig. 2).

From the curves it is readily seen that the unground coagulated oval-

bumin is digested at the most rapid rate. The ground coagulated ovalbumin, from which the water soluble protein has been extracted is digested at a much slower rate indicating differences in the two insoluble proteins. The water insoluble protein produced by grinding native ovalbumin for 24 hours is digested at a slower rate than the heat coagulated egg albumin which has been ground for two 48 hour periods and extracted free of water soluble protein. The ground insoluble acid denatured ovalbumin is digested at a slower rate than heat coagulated ovalbumin but is digested at almost the same rate as the ground insoluble native ovalbumin during the first 20 hours. The acid denatured ovalbumin which has been ground for 36 hours and is insoluble at pH 3.0 is digested more rapidly than the unground acid denatured ovalbumin.

DISCUSSION

It has been mentioned that the coagulated ovalbumin after grinding acquires a definite yellow coloration and a peculiar characteristic odor. This is true of all the proteins studied up to this time and may be due to decomposition of the aromatic amino acids.

The grinding of native ovalbumin brought about definite irreversible changes in the protein molecule. This change is evidenced by the conversion of the water soluble protein into one which is insoluble in water. Since some of the ground water insoluble protein is soluble in acid (pH 3.0) and alkali (pH 9.5) and the major portion is insoluble, it would seem that the conversion occurs in at least two stages. The first is a transformation of the water soluble protein into a protein soluble in acids and alkali, not unlike a denatured protein. The second stage is the transformation into a highly insoluble protein resembling coagulated ovalbumin in its solubilities.

The grinding of acid denatured ovalbumin brings about definite changes in the molecule. After grinding the powder contains little protein soluble in acid and alkali. This might possibly be explained on the assumption that the changes are merely intramolecular in character, involving the weaker secondary valence bonds.

The dry grinding of coagulated ovalbumin breaks down this insoluble protein so that water soluble protein results. The water soluble protein obtained after grinding heat coagulated ovalbumin contains a higher percentage of sulfur, a slightly lower nitrogen content and a lower tryptophan and tyrosine content than the native ovalbumin. The water

soluble fraction, split off during the first 48 hours of grinding, contains no tryptophan.

The electrophoretic analysis indicates that there may be a stepwise degradation of the protein produced by the grinding process. It would appear from the results that the effect of dry grinding has been to produce a most decided disintegration of the protein material.

SUMMARY

1. The dry grinding of native crystallized ovalbumin yields a highly insoluble protein somewhat resembling coagulated ovalbumin.

2. Grinding acid denatured ovalbumin produces an acid (pH 3.0) insoluble protein.

3. The grinding of heat coagulated ovalbumin produces water soluble protein.

4. The sulfur, nitrogen, tryptophan and tyrosine content of these water soluble fractions has been determined.

5. Electrophoretic analysis of the water soluble fractions from heat coagulated ovalbumin indicates that there may be a stepwise degradation of the protein produced by the grinding process.

6. The results indicate a decided disintegration of the protein molecule.

7. The digestion rates of native, ground native, coagulated, ground coagulated, denatured and ground denatured ovalbumin have been compared.

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The Action of Liver Extracts in Counteracting the Toxic Effects of Diethylstilbestrol and Sulfanilamide

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In 1922 one of us (C. F.) (1) expressed the view, based on the papers of Reid Hunt and Salant, that the composition of the diet and perhaps its vitamin content may have a profound influence on the toxicity of drugs. C. Funk and I. C. Funk (2) have shown that diethylstilbestrol is rendered less toxic when the rat food is fortified with 10 per cent of whole wheat.

In the present study we have continued the investigation of the detoxication of diethylstilbestrol. A large number of rats was used in this study and the animals were placed on a standard Purina diet for two weeks prior to injections. First, we determined the dose of the drug which killed about 50 per cent of the animals within 1-2 days. Having determined the dose to be 650 mg. per kg. intraperitoneally we studied the detoxifying effect of certain nutritive ingredients which could be found in a whole wheat diet. We concentrated our efforts on the action of B₁, nicotinic acid amide and whole liver extract. These products were injected either separately or in combination and after a number of experiments it was concluded that whole liver extract diminished the toxicity of the drug, perhaps even better than the combination mentioned above.

Having found whole liver extract highly valuable in diminishing

¹ We wish to thank Dr. G. C. Scherf, Medical Superintendent, Goldwater Memorial Hospital for his helpful cooperation. We also wish to thank Miss Mary Bell, Bacteriologist of Metropolitan Hospital, New York City for her helpful advice, and Miss Florence Meisner, of the Goldwater Memorial Hospital for assistance in the hematological studies. One of us (C. F.) gratefully acknowledges the receipt of a research grant from the U. S. Vitamin Corp. of New York City.

the toxicity of diethylstilbestrol, similar experiments were conducted with sulfanilamide. Here, also, the toxic dose was first determined for rats. It was found that 400 mg. per kg. intraperitoneally killed about 50 per cent of the animals in 1-2 days. The detoxication experiments were performed both with whole liver extract and an injectable preparation of B complex factors from yeast. The dose of yeast extract used was calculated to conform with the dose of liver extract injected. Whole liver extract appears to be superior to yeast as a detoxifying material.

In view of the fact that Rosenthal (3) and others have found that a high protein diet, *p*-aminobenzoic acid and a number of other substances impede the bacteriostatic action of sulfa drugs, and, because such substances might be present in whole liver extract, special care was taken to determine whether the detoxifying action of the liver extract was not due to inactivation of the sulfa drug. In doing so we encountered serious difficulties. We were unable to secure a bacterial culture which was lethal for rats. We had to use mice, determine the toxic dose of the sulfa drug, the lethal dose of the bacterial suspension, etc. Finally, the proper conditions were determined and we found that the survival of the mice on the combination of sulfa drug and liver extract was even better than with the drug alone.

We shall not, for the present, discuss the mechanism of the toxic action of sulfa drugs and of nature of substances in the liver which prevent these biochemical disturbances. During the present studies papers have appeared, which deal with the subject. Schaefer, McKibbin, and Elvehjem (4) have found that sulfapyridine interferes with the curative action of nicotinic acid in dogs Black Tongue and that this action is restored with whole liver, but not its extracts. Later, Nielsen and Elvehjem (5) fed 1 per cent succinylsulfathiazole, added to the diet over a comparatively long period of time. It arrested growth, which was restored by folic acid and biotin administration. Similar technic was used by Daft, Ashburn, and Sebrell (6). These investigators have found that sulfa drugs produce biotin deficiency and various pathological changes, which were minutely described. The liver extracts, used by the last named authors, were found markedly detoxifying. The experiments of the authors mentioned under (4, 5, 6) differ from ours in that they concern chronic intoxication by sulfa drugs, while our experiments deal with acute toxicity, which did not produce chronic pathological changes. We agree with these authors that liver possesses a highly detoxifying capacity for sulfa drugs.

TABLE I

Influencing the Toxicity of Diethylstilbestrol with a Mixture of B₁, Nicotinamide and Liver Extract, as Contrasted with Liver Extract Alone

Detoxicant used	Males				Females			
	Number of rats injected	Number of rats dead	Initial weight average	Weight changes in per cent	Number of rats injected	Number of rats dead	Initial weight average	Weight changes in per cent
I. Drug alone 650 mg. kg.	16	8	135	-2.4	16	8	133	-2.0
II. 5 mg. B ₁ , 20 mg. Nicotinamide, 100 mg. liver BX/kg.	16	4	118	-2.2	16	0	113	
III. Same mixture as II but liver 20:1	18	4	156	-1.6	18	4	143	
IV. Same mixture as III, but liver increased to 250 mg. 'kg.'	6	4	131	-2.5	6	3	137	-6.8
Liver extract alone and Sulfa drug								
V. Liver 20:1 100 mg./kg.	18	4	149	+1.4	18	2	144	+0.5
VI. Same liver as V, but but incr. to 250 mg./kg.*	6	3	148	+4.0	6	2	136	-3.5
VII. Liver BX 100 mg./kg.	6	4	206		6	1	204	0

* Increased liver dosage appears to be harmful.

The liver extract designated as 20:1 was the total liver extract of Wilson Laboratories, while the one designated as BX was the secondary anemia fraction of Wilson Laboratories. While in the Exp. II the weight of the animals happened to be lower, it was found that the toxicity of diethylstilbestrol is practically independent of the weight of the animals, the dose of 650 mg./kg. killing ca. 50 per cent of the rats. Except for the higher liver dosage (250 mg./kg.) the results are quite consistent and indicate that a mixture of B₁, nicotinamide and liver extract diminishes the toxicity of diethylstilbestrol. Better results were obtained with liver alone in moderate doses. In the above experiments diethylstilbestrol shows 50 per cent higher toxicity in males, judging by mortality. By the weight depression, however, the toxicity is higher in surviving females.

Four females injected with 1 mg. of diethylstilbestrol and 100 mg./kg. liver have shown that the estrogenic activity remains unimpaired by liver administration.

In summarizing our results with diethylstilbestrol, based on all the animals used, including those to determine the toxic dose and not shown in Table I, we conclude that whereas the dose of 650 mg. per kg. weight kills about 50 per cent of the rats of various age and weight groups, the simultaneous administration of total liver extract reduces the mortality to about 25 per cent.

We wish to emphasize the significant fact shown in the present studies that the toxicity of two drugs so different in their chemical structure and pharmacological action, as diethylstilbestrol and sulfanilamide,

TABLE II

Influencing the Toxicity of Sulfanilamide with Total Liver and Yeast Extracts

Detoxicant used	Males				Females			
	Number of rats injected	Number of rats dead	Initial weight average	Weight changes in per cent	Number of rats injected	Number of rats dead	Initial weight average	Weight changes in per cent
I. Drug alone 400 mg./kg. . . .	12	7	124	-1.4	12	6	122	-1.5
II. Drug and liver 20:1 100 mg./kg.	12	3	135	+1.8	12	3	127	+2.3
III. Drug along 400 mg./kg.	6	3	217	-3.6	6	4	202	
IV. Drug and liver 20:1 100 mg./kg.	6	1	206	-1.0	6	2	162	0
V. Drug alone 400 mg./kg.	8	2	118	-4.0	8	6	124	
VI. Drug and yeast extr. 650 mg./kg.	6	3	136	-7.3	6	2	140	+2.9
VII. Drug and same extr. as VI 250 mg./kg.	6	2	203	+1.3	6	2	177	+1.9

The sulfa drug was injected at a level of 400 mg. kg. which kills approximately half of the number of animals, practically independent of weight, age and sex. The yeast extract was Vico Brand Yeast Extract or no. 200-R and was prepared for injection in the same way as the liver extract. The results with total yeast extract suggest some degree of activity, but are not yet conclusive.

Of the 52 rats of both sexes injected with sulfanilamide alone 28 died, while with drug supplemented with liver extract, out of 36 animals only 9 died. Summarizing the results of our studies with liver extract in conjunction with diethylstilbestrol and sulfanilamide we found the mortality dropping from 50 per cent to about 25 per cent.

should be so markedly influenced by the same agent, a whole liver extract.

EXPERIMENTAL

For the toxicity test an equal number of male and female rats (Wistar strain) were used. The experimental groups were chosen to be as uniform as possible regarding age and weight, although these factors were

found later to have practically no bearing on the results in the range of our experimental animals. The animals were placed on a standard Purina laboratory diet for two weeks prior to the tests to exclude the influence of diet variations. The animals were injected intraperitoneally and kept under close observation for a period of 5 days. The degree of toxicity was determined by the mortality and the change in weight of the survivors. The diethylstilbestrol was injected in a 10 per cent solution in olive oil. The liver extract was adjusted with alkali to pH 8.0, the insoluble residue separated by centrifuging and the clear solution was used.

Having found that the liver extract greatly diminishes the toxicity of sulfanilamide, it was necessary to establish, whether this action interferes with the bacteriostatic action of the drug. Unable to find a suitable bacterial culture for rats, we had to turn our attention to mice, where we could profit from the considerable experience of earlier workers.

Three separate experiments were performed using mice of 25-30 g. weight. As they gave identical results, only the last experiment will be recorded in detail. Here 12 mice were used in each group. A saline suspension of a 48 hour culture of *Streptococcus haemolyticus* in dilution 10^6 was used, 0.3 ml. of the suspension being injected intraperitoneally into each animal. Sulfanilamide in a dose of 150 mg./kg. and liver extract 20:1 in a dose of 100 mg./kg. were injected in the corresponding groups. It was found necessary to repeat the injections of the sulfa drug and liver extract in same dose 24 and 48 hours after the initial injection. The following table summarizes the results:

Strept. haemol. Strept. and sulfa drug Strept. Sulfa drug Liver extr. Strept. and liver extr.

	Strept. haemol.	Strept. and Sulfa Drug	Strept., Sulfa Drug and Liver Extr.	Strept. and Liver Extr.
Average survival in hours	44	337	392	57
Range of hours	30-47	318-370	348-416	48-60

To render these results more significant differential and leucocyte counts were made on 3 animals of each group, every 8 hours, taking the blood from the tail. The changes observed reached a peak after 16 hours, later approaching slowly the normal count. Therefore, the 16 hour counts will be here recorded.

	I. Normal	II. Strept.	III. Strept. and Sulfa Drug	IV. Strept., Sulfa and Liver Extr.	V. Strept. and Liver Extr.
Leucocytes	13,900	19,350	7,200	11,100	18,700
Polynucl.					
immature	4	6	4	10	5
mature	16	28	34	19	24
Lymphocytes	76	66	62	69	71
Monocytes	4	0	0	2	0

It appears significant that the leucocyte count in the Sulfa group (group III) returned to normal within 120 hours, whereas in the group IV (with liver) in 72 hours. On the whole, the experiments on mice corroborate our results on rats. The liver extract not only does not inactivate the bacteriostatic power of the sulfa drug, but, on the contrary, appears to be beneficial. Both the time of survival and the blood counts point to this conclusion. The total number of animals used in the present paper was 402 rats and 225 mice.

SUMMARY

1. The administration of 650 mg./kg. of diethylstilbestrol and of 400 mg./kg. of sulfanilamide to rats, both by injection, causes a mortality of about 50 per cent in 1-2 days, usually in the first 24 hours. The toxicity appears to be practically independent of age and weight, and in case of the last drug, also of sex.

2. Simultaneous administration of total liver extract in a dose of 100 mg./kg. together with both drugs, diminishes the mortality from 50 per cent to 25 per cent and diminishes the weight losses in the surviving animals. It appears significant that liver extract acts with both drugs, in spite of their different chemical structure, in an analogous way.

3. Experiments on mice with *Streptococcus haemolyticus* corroborate the rat experiments. Here also the liver extract diminishes the toxicity of the sulfa drug, without impairing the bacteriostatic action. On the contrary, both the survival time and the results of the blood counts indicate a beneficial action of the liver extract.

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Studies on the Kinetics of Cell Respiration, IX.
Acceleration of Pyruvate Oxidation in *Escherichia coli* by
Certain Amino Acids, Ammonia, and 4-Carbon
Dicarboxylic Acids

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INTRODUCTION

Our knowledge of the intermediate steps in carbohydrate metabolism has advanced considerably in recent years, but comparatively little is known regarding the oxidative breakdown of amino acids by the cell. A number of possible routes exists for the aerobic and anaerobic deamination of an amino acid, and this has been summarized by Stephenson (1; pp. 35 *et seq.*) who concludes that the mode of oxidative breakdown of the amino acids is still obscure. The experiments reported in this account were conducted as an attempt towards the elucidation of this problem.

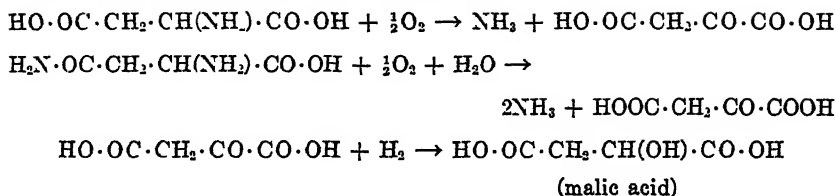
Preliminary experiments showed that *Escherichia coli* can utilize certain amino acids as well as pyruvic acid. Furthermore the oxidation of pyruvate by *E. coli* may be accelerated not only by the 4-carbon dicarboxylic acids such as succinic, fumaric, and malic but also by ammonia and by certain amino acids. The amino acids tested were: serine, aspartic acid, glutamic acid, hydroxyproline, cystine, phenylalanine, arginine, and asparagine. Only the first three amino acids, and to a lesser extent the amide asparagine, are oxidized by *E. coli*, but the rates of oxidation of any of these compounds alone are much slower than when they are added to pyruvic acid.

We may consider the aerobic breakdown of the three amino acids in the following manner:

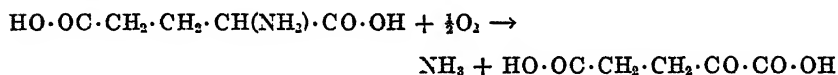
For serine:



For aspartic acid (and for asparagine):



For glutamic acid:



And we may consider that the breakdown products, ammonia and the dicarboxylic acids, are responsible for the accelerating effect exhibited by the amino acids.

In order to prove these points, it is necessary to demonstrate that: (A) Both ammonia and malic (succinic or fumaric) acid are able to accelerate pyruvate oxidation, and that the effect is additive and catalytic. (B) Acceleration by serine should be less than that due to glutamate or aspartate, since the first compound yields only one active substance, ammonia, whereas the last two yield both ammonia and a dicarboxylic acid. (C) The respiratory quotients for the oxidation of all three amino acids are zero, since no carbon dioxide is given off during the process. Should the respiratory quotient of any of the compounds be finite, it indicates the decarboxylation of the keto acids in the above equations.

EXPERIMENTAL

The organism used in these experiments was a culture of *E. coli* kindly supplied to us by the Central Epidemic Prevention Bureau, as was also that used in a previous experiment of this series (2). The cells were cultured in beef extract at 35° C. A 24-hour culture was used in each of the experiments, centrifuged three times, being resuspended after each centrifugation in a phosphate buffer of pH 7. Preliminary experiments showed that with this treatment, without added substrate, the rate of oxygen uptake is negligible for such short periods as those used in these experiments. Two ml. of the suspension were pipetted into each of the conical Warburg vessels, with or without pyruvate or other substrate. When substrates are added they are introduced in

0.5 ml. quantities, either to the main vessel or to the sidearm as the case demands. A few experiments were performed on the respiratory quotients of the cells. In these experiments the vessels were used in pairs. Into the well of one vessel was introduced the usual 0.2 ml. of 10 per cent NaOH, while the well of the other contained the same quantity of ten per cent HCl for absorption of ammonia which might be produced in experiments with amino acids. The vessels were kept at 35° C. and shaken at approximately 70 oscillations per minute for the duration of the experiment which usually lasted for 2 hours.

All the organic chemicals used were from Eastman Kodak Co., except citric acid and sodium glutamate which were purchased locally. The source of ammonia is C.P. ammonium chloride.

RESULTS

The results of the experiments are summarized in Table I. In that table the rates of oxygen consumption for *E. coli* (a) in pyruvate alone, and (b) in pyruvate plus amino acids and other acids or ammonium chloride, are given, together with the percentages of acceleration. It is seen that glutamate accelerates pyruvate oxidation to 240 per cent. Aspartic acid accelerates to 190 per cent, while serine stimulates pyruvate oxidation to only 63 per cent. The amide asparagine, although possessing 2 amino groups, is only about as effective as aspartic acid. Arginine, which possesses 4 potential amino groups, gives only 80 per cent acceleration.

The fact that all three 4-carbon dicarboxylic acids accelerated the rate of pyruvate oxidation is to be expected from the work of Szent-Gyorgyi (3). In the case of citric acid, which according to Krebs should behave like the other dicarboxylic acids, we observed an accelerating effect amounting only to 35 per cent. Among the 4-carbon acids, malate was the most effective in accelerating pyruvate oxidation. This was followed by succinate. In the case of fumarate, there was a lag period of about 60 minutes after which an accelerating effect was observed. This was perhaps due to the conversion of fumarate into either malate or succinate which are immediately concerned in the acceleration process. In the table two values are given for the rate of pyruvate oxidation with added fumarate. The first value of 58 was obtained during the first 60-minute period, and the second, 71, which was 26 per cent higher than the rate in pyruvate alone, was that for the second period. The rate in ammonia plus pyruvate was 64 per cent higher than that in

Rate of O₂ Consumption (ml. hr⁻¹) by E. Coli in Presence of Pyruvate Alone and in Presence of Pyruvate Plus Other Substrates

Duration of experiments, 2 to 3 hrs.; temp. 35°C, pH 7.0, concentration of all substrates 1/10.

Substrate	ml. O ₂ consumed per hour	Differ- ence	Per cent acceleration
1. Pyruvate	88		
2. Pyruvate + aspartate	255	167	190
3. Pyruvate	80		
4. Pyruvate + arginine	147	67	84
5. Pyruvate	70		
6. Pyruvate + serine	114	44	63
7. Pyruvate	67		
8. Pyruvate + glutamate	228	161	240
9. Pyruvate	295		
10. Pyruvate + phenylalanine	225	-70	
11. Pyruvate	83		
12. Pyruvate + asparagine	166 [*]	83	100
13. Pyruvate	80		
14. Pyruvate + hydroxy proline	90	10	12.5
15. Pyruvate	22		
16. Pyruvate + cystine	27	5	23
17. Pyruvate	100		
18. Pyruvate + NH ₄ Cl	164	64	64
19. Pyruvate	54		
20. Pyruvate + malate	91	37	68.5
21. Pyruvate	45		
22. Pyruvate + succinate	65	20	44.5
23. Pyruvate	54		
24. Pyruvate + fumarate	(58 ¹ , ** 71)	17	32
25. Pyruvate	114		
26. Pyruvate + citrate	153	39	34
27. Pyruvate	225		
28. Pyruvate + pyruvate (control).	214	-11	

pyruvate alone, and this may account for the acceleration due to serine, which amounted to 63 per cent. When more pyruvate was added to the pyruvate control, no acceleration was observed, showing that acceleration due to the added substrates was not due to a concentration effect.

It is interesting to note that both ammonia and the 4-carbon acids accelerated pyruvate oxidation by *E. coli*, but the percentages of acceleration were much lower than those for glutamate and aspartate. In the case of serine, the percentage of acceleration was about the same as that for ammonia. In order to test whether the effect of aspartate on pyruvate oxidation was due to the additive effect of ammonia and of the 4-carbon acids, experiments were performed with the same cell suspension, one, using aspartate plus pyruvate, another, pyruvate plus ammonia, and a third, malate plus pyruvate. In one experiment, with a 5-day culture, the percentages of acceleration were as follows: for pyruvate plus aspartate, 53.3; for pyruvate plus ammonia, 17.3; and for pyruvate plus malate, 36. In another experiment, with a 24-hour culture, the percentages were, respectively, 103; 74; and 34. In each case, the sum of the last two percentages of acceleration is equal or nearly equal to the first, that due to pyruvate plus aspartate, indicating that aspartate acceleration was due to the combined effect of ammonia and malate (or succinate). The high percentage of acceleration due to glutamate was not subjected to a similar analysis since (a), the compound was obtained on the local market and its purity was not certain, and (b), as will be shown below, it is probable that glutaric instead of succinic acid is formed in the breakdown of this compound.

That the effect of aspartate on pyruvate oxidation is at least partly catalytic is shown by the following experiments: The addition of 0.5 ml. of $M/10$, $M/20$, $M/50$, $M/100$ or $M/200$ aspartate to cells suspended in 2 ml. of phosphate buffer pH 7 and 0.5 ml. of $M/10$ pyruvate gives the same extent of acceleration. In another experiment, 0.5 ml. of a $M/200$ aspartate was added to 2.5 ml. of cell suspension. This amount of aspartate, if oxidized along with pyruvate, would consume approximately 80 ml. of oxygen (at 35°C., 610 mm.) in excess of that for the cells suspended in pyruvate alone. Actually, at the end of 2 hours, the excess of oxygen consumed was 183 ml., which is more than twice the amount calculated, and if the experiment had been continued for a longer time, the excess oxygen consumed would have been much more than 183 ml.

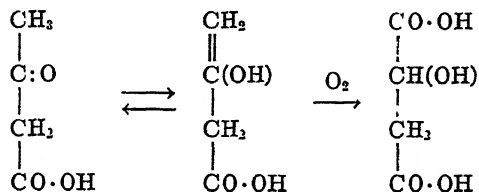
The respiratory quotients, as measured with the differential method of Warburg, using HCl in the well instead of NaOH in the vessel for $\text{CO}_2\text{-O}_2$ determination were, for serine and glutamate, zero, showing no decarboxylation in the process. The value for pyruvate oxidation and aspartate oxidation was 0.5 for several experiments. Taking the R.Q. values of the three amino acids as given above, we may infer that the aerobic breakdown of serine results in the formation of ammonia and α -keto- β -hydroxy propionic acid. The breakdown products of glutamate are ammonia and keto-glutaric acid. And the breakdown of aspartic acid gives rise to ammonia, keto-succinic or malic acid, which in turn may be decarboxylated and eventually give rise to $\text{HO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{OH}$, or perhaps lactic and pyruvic acids. It is unlikely that malonic acid is formed in the process since it is known that this acid inhibits tissue respiration (4).

DISCUSSION AND CONCLUSIONS

Harden (5), as long ago as 1901, found succinic acid to be the product of aspartic acid-putrefaction by *E. coli*. In alcoholic fermentation, Ehrlich (6) failed to confirm this finding but attributed the source of succinic acid to glutamic acid. Recently Örstrom (7) demonstrated the stimulation of the respiration of the eggs of *Paracentrotus lividus* by ammonia. Since the three 4-carbon acids are mutually convertible, it is immaterial which one is postulated as the intermediate step of aspartate breakdown. In view of the very wide difference existing between the effect of glutamate on pyruvate oxidation on the one hand and that of malate, succinate and ammonia on the other, and in view of the absence of decarboxylation for glutamate oxidation, it is quite probable that keto-glutaric instead of succinic acid is the product of glutamate breakdown in *E. coli* oxidation. The accelerating effect of ammonia is obscure, and this will be the subject of a subsequent investigation.

The significance of the experiments reported in this account is the demonstration of the acceleration of pyruvate oxidation in *E. coli* by the three amino acids and asparagine, the demonstration of the fact this effect is due to the combined action of ammonia and of malate (succinate, or fumarate), which are the breakdown products of these amino acids, and finally the establishment of the probable courses of oxidative breakdown for these amino acids as indicated in the three schemes given at the beginning of the account. The result for glutamate should, however, be taken with a certain degree of reserve, as the chemical used may not have been as pure as desired.

In view of these experiments, it is perhaps relevant to add a few words regarding the cause of the specific dynamic action of amino acids in animals. It is probable that the phenomenon is due to the combined effect of ammonia and the fatty acid residue arising from the breakdown of amino acids, and that the stimulation of metabolism is due to ammonia on the one hand, and such dicarboxylic acids as malic and glutaric on the other. It is true that in man the amino acids most effective for specific dynamic action are: glycine, alanine, tyrosine, and leucine, but these are able to give rise to acetoacetic acid, which, when the terminal carbon is oxidized, may give rise to malic acid in the following manner:



SUMMARY

Oxidation of pyruvate by *E. coli* is catalytically accelerated by the addition of aspartic acid, glutamic acid, serine, or asparagine. The acceleration is brought about by ammonia and dicarboxylic acids. Probable courses for aerobic deamination of these amino acids are thus established, and the bearing of these findings on the cause of specific dynamic action of the amino acids is discussed.

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The Isolation of Hexenal from Leaves

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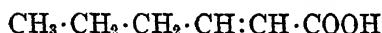
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INTRODUCTION

As a product of the steam distillation of leaves, the straight chain, six carbon atom aldehyde, hexenal, has been obtained from a large number of plant species. The apparent wide distribution in higher plants of this aldehyde has been variously interpreted. It was thought to be associated with the photosynthetic process by Reinke (1, 2, 3) and by Meyer (4), while Curtius and Franzen (5) proposed the idea of its being an intermediate step in the conversion of sugars to fats. Takei and coworkers (6, 7, 8) associated the characteristic odor of freshly cut grass with that of hexenal, and also presented evidence that this aldehyde together with the corresponding alcohol were responsible for the aroma of tea. Before any of these divergent ideas can be subjected to further experimental tests, it is obviously essential to establish whether the compound in question is actually a component of the living plant, or whether perchance it is an artifact, produced during the drastic treatment to which the plant material is exposed in the process of extraction. It is to this aspect of the problem that the investigations here reported have been directed.

Curtius and Franzen (5) subjected 20-kilogram lots of ground leaves to steam distillation and in the distillate precipitated the hexenal with *m*-nitrobenzhydrazide. After recrystallization, the precipitate had a melting point of 167–168° and an empirical formula $C_{13}H_{15}N_3O_3$. The free aldehyde was obtained by distillation of the condensation product with sulfuric acid. The distillate combined again with *m*-nitrobenzhydrazide to form a product identical with the original, it condensed also with benzhydrazide to give a compound melting at 108–109°, and with *p*-nitrophenylhydrazine to give a hydrazone melting at 137°. On oxida-

tion with silver oxide the aldehyde gave an acid identical with that to which Fittig and Baker (9) assigned the formula



obtained by boiling hydrosorbic acid with potassium hydroxide. The calculated molecular refraction based on the formula



corresponded closely to that found experimentally.

The amount of condensation product with *m*-nitrobenzhydrazide was determined by Curtius and Franzen in the steam-distillate of 20 different species of leaves. An exhaustive study of the steam-volatile oils of the leaves of sixteen species of plants with regard to their hexenol and hexenal content has been made by Takei and co-workers (6). In the case of tea very large quantities of leaves were used for steam-distillation, and the yields were extremely small: 137.76 g. hexenol and 2.29 g. hexenal from 1861 kg. of the fresh leaves of *Thea sinensis*, and 85.62 g. hexenol and 4.29 g. hexenal from 1947 kg. "green tea" of the same species. From the investigations of Curtius and Franzen (5) the amount of hexenal obtained from the steam-distillates of leaves of various species varied from 20 to 500 mg. of the *m*-nitrobenzhydrazide condensation product per kilogram of fresh leaves.

MATERIAL AND METHODS

None of the species of plants which previous investigators used was available to us in sufficient quantity for extensive experimentation. A preliminary survey of hexenal obtainable from available plant material, in which 15 species of plants were studied, revealed that *Ailanthus glandulosa* (Chinese tree of Heaven) gave the best yields of the condensation product, in a fair degree of purity. The fresh leaves were locally available during much of the year. Although *Ailanthus* is a good source of hexenal on the whole, the amount of *m*-nitrobenzhydrazide condensation product obtained from different samples of leaves receiving the same treatment ranged from 0-450 mg. per kilogram of fresh leaves. This yield is apparently affected by a number of factors such as the season, light conditions and temperature under which the plants have grown. Any comparison concerning the effect of one known imposed variable must, therefore, be made relative to a defined control.

The aldehyde obtained from leaves by steam distillation was compared with a synthetic preparation of hexenal. For this purpose 2-hexenal was synthesized according to the method of Dellaby and Guillot-Allegre (10). The index of refraction of the synthetic 2-hexenal for the sodium line was 1.4424 at 22°, which compares favorably with the value given by Curtius and Franzen of 1.4460 at 17.9° for the aldehyde they obtained from leaves. The synthesized 2-hexenal *m*-nitrobenzhydrazide melted at 164° (uncor.). The melting point of the recrystallized *m*-nitrobenzhydrazide from leaves melted at 164°. This product, when mixed with the *m*-nitrobenzhydrazide of 2-hexenal also melted at 164°.

It must be realized that *m*-nitrobenzhydrazide as a quantitative reagent for the determination of hexenal has definite limitations. Special experiments showed that the solubility of the condensation product under the conditions here used ranges from 15 to 22 mg. per 100 ml. of water. Since, in the method used, 500 ml. of distillate is equivalent to 1 kg. of leaves, an addition of 75 to 100 mg. would have to be made to the yields reported in the following sections in the cases in which a precipitate was obtained from the steam distillate. In the cases in which no precipitate was obtained, it is possible that the true yield may have been as much as 75 mg. per kg. of leaves. These corrections were, however, not applied in the results hereinafter reported.

A weighed quantity of leaves (500 g. to 1 kg.) was ground in a meat grinder, mixed with 2 liters of water in a 5-liter round-bottom flask, and attached to a steam generator and a condenser. The mash was distilled until 50 ml. of distillate per 100 g. of leaf material had been collected. For every 100 ml. of distillate obtained, 10 ml. of 95% ethanol containing 100 mg. of *m*-nitrobenzhydrazide was added. The mixture was allowed to stand for 24 hours, filtered on a hardened filter, dried in vacuo over CaCl_2 and weighed. The yield of the aldehyde obtained from a leaf distillate was expressed in milligrams of aldehyde condensation products per kilogram of fresh leaf material.

Repeated experiments demonstrated that the yield of aldehyde condensation product was materially affected by the process of grinding the leaves prior to steam distillation. In order to shorten the period of grinding and to facilitate this process the leaves were put through a green-feed cutter prior to grinding. This process chopped the leaves into 3 mm. strips and reduced the long fibers which rendered grinding difficult. The effect on the yield of aldehyde of mincing the leaves

prior to steam distillation may be illustrated by the results of an experiment carried out as just described. One portion of leaves was chopped, ground, and the mixture with 2,000 ml. of hot water, was distilled with steam; another portion of leaves was steam distilled in exactly the same manner, but without having subjected the leaves to any mincing processes. From the portion which had been ground a yield of 333 mg. of hexenal condensation product per kg. of leaves was obtained, while the leaves which had not been ground yielded no detectable amount of condensation product.

Without recording here the details of other experiments of this nature, the results may be summarized: that leaves which were chopped and ground before distillation gave a much larger yield of aldehyde than did leaves which were only chopped and then distilled, while leaves which were distilled without chopping or grinding gave no precipitate.

Two explanations suggest themselves to account for this effect: 1. the aldehyde may be present in the whole leaves, but is released only after the leaves are ground, or 2. the aldehyde is formed during the grinding process. If the aldehyde were formed during the grinding process this might be a vital or enzymatic reaction, possibly involving atmospheric oxygen. In case an enzymatic reaction were involved the formation of aldehyde would be inhibited by heat, and, in case oxygen played a rôle, the formation of the aldehyde would probably be inhibited by grinding in an inert atmosphere.

EXPERIMENTS

1. *Effect of Heat*

If the formation of the aldehyde were due to an enzymatic reaction occurring during the grinding process it should be inhibited by heat, and leaves which were subjected to temperature of boiling water before grinding should produce no more aldehyde than leaves which were distilled without grinding. Results demonstrating that the formation of the aldehyde is inhibited by heat are shown in the following experiments. Leaves which were treated with hot water prior to chopping and grinding, (A) gave no aldehyde. Leaves which were treated with hot water after they had been chopped but before they had been ground, (B), gave only a small amount, as compared with leaves which were treated with hot water after they had been chopped and ground (C) and (D).

3.9 kg. of *Ailanthus* leaves were divided into 4 portions:

A. 0.9 kg. of leaves were placed in 2,000 ml. of boiling water. The water was drained off, the leaves were *chopped* and *ground*, the water which had been used to kill the leaves was added to the ground leaves and the mixture was distilled with steam. Yield: none.

B. 0.9 kg. of leaves were *chopped* and placed in 2,000 ml. of boiling water. The water was drained off, the leaves were *ground*, and together with the water used for killing, was steam distilled. Yield: 3 mg. kg.

C. 1.1 kg. of leaves were *chopped*, *ground* and placed in 2,000 ml. of boiling water. The water was drained off, again added to the leaves, and the whole mixture subjected to steam distillation. Yield: 159 mg. kg.

D. 1.0 kg. of leaves was *chopped*, *ground* and distilled together with 2,000 ml. of water. Yield: 168 mg. kg.

From the results of the foregoing experiment it seemed very improbable that the aldehyde was present in detectable amounts in the whole leaves. Yet in the cases in which the leaves were killed before grinding the aldehyde may have been mechanically prevented from escaping. Special experiments were carried out to test this point. Comparative determinations of the aldehyde from the same lot of leaves were made by first killing the leaves before grinding, with hot water, or with a hot 5 per cent solution of NaH_2PO_4 or with a hot 5 per cent solution of H_3PO_4 . The NaH_2PO_4 and H_3PO_4 were used to effect a more complete maceration of the leaf cells during the process of steam distillation. However, no significant differences in the amount of aldehyde condensation product were obtained by the use of these killing agents.

2. The Half-leaf Method

The fact has already been mentioned that although considerable quantities of hexenal condensation product are obtainable from the leaves of *Ailanthus* by distillation with steam, the yields vary considerably according to the seasonal and daily changes of the environment in which the plants are growing. In order to simplify this problem and to obtain material which gave reproducible results at least within one series of experiments, advantage was taken of the fact that *Ailanthus* has a large compound leaf consisting of six to twelve pairs of leaflets, arranged opposite along the midrib. This permits the stripping of the leaflets from one side to be used as a control, and using the leaflets from the other side in experiments in which a variable is imposed. By this method the two opposite halves of the compound leaf, each consisting

of 580 g. of leaflets, yielded 302 mg. and 338 mg. of the condensation product per kg. of fresh leaves.

3. Effect of Irrespirable Gases

That oxygen is involved in the formation of the aldehyde during the grinding process of the leaves becomes evident from the following experiments. In these, comparative determinations of the aldehyde were made by means of the half leaf method, just described. Leaflets from one side of the leaf were ground in air and the amount of aldehyde condensation product obtained from this was compared with that obtained by grinding the leaflets from the other side of the leaf in an atmosphere of nitrogen or carbon dioxide.

A. 0.6 kg. of leaflets from one side of *Ailanthus* leaves were *chopped*, ground and distilled with steam. Yield: 90 mg. kg.

B. 0.6 kg. of leaflets from the other side of the same leaves used in A. were *chopped*, and placed in a glass bacteria mill with stainless steel balls and 600 ml. of cold water. The mill was evacuated four times and filled with carbon dioxide after each evacuation. The leaflets were ground in an atmosphere of carbon dioxide for 2 hours by rotating the mill on rollers. The contents of the mill, after addition of hot water, were subjected to steam distillation in a flask. Yield: 5 mg./kg.

4. Effect of Fine Grinding

In our preliminary experiments it had been noticed that the yield of hexenal even from the same lot of leaves varied considerably and that this was apparently dependent upon the thoroughness with which the leaves were ground. By grinding the leaves in a bacteria mill very much more thorough disintegration was obtained than by grinding in a meat grinder. This fineness of grinding is also reflected in the yield of hexenal obtained as is illustrated in the following experiment.

A. 0.63 kg. of leaflets from one side of the leaves were *chopped*, placed in a bacteria mill with stainless steel balls and 25 g. of sand with 500 ml. of water and rotated for 2 hours. A manometer connected with the bacteria mill, which had a capacity of 2,000 ml. indicated that the gas pressure (air) had been reduced 12 mm. of mercury during the grinding. The thoroughly ground mass was transferred to a distilling flask, 1,500 ml. water added, and distilled with steam. Yield: 417 mg., kg.

B. 0.64 kg. of leaflets from the opposite side of the same leaves used in A. were *chopped* and ground in a meat grinder. The mass was distilled with steam in the same manner. Yield: 89 mg. kg.

5. Distillation in an Inert Atmosphere

From the foregoing experiments the conclusion seems warranted that hexenal is formed by the exposure of the injured leaf tissue to oxygen, and that this oxidation takes place only in the presence of a component of the leaf which is inactivated by heat, *i.e.* a component presumably of enzymatic nature. In order to test these conclusions more critically, it seemed desirable to carry out experiments in an apparatus in which the leaves could be ground in an inert atmosphere and in which the ground tissue could then be subjected to steam distillation without exposure to air. To prevent any oxidative changes during the heating prior to distillation and while the distillate was in the vapor state, an apparatus was arranged which permitted the distillation also to be carried out in an inert atmosphere.

The apparatus for grinding leaves in an inert atmosphere consisted essentially of a cylinder, closed at one end, and a removable cover, with inlet and outlet tubes. The cylinder was constructed of 8 inch well casing, $\frac{3}{16}$ inch in thickness. To the center of the closed end a pipe was fastened, extending along the axis of the cylinder and through a hole in the cover. This pipe, $\frac{1}{2}$ inch in diameter, had four holes about one-half inch from where it was fastened to the closed end of the cylinder. To the open end of the cylinder an annulus, 6 inches internal diameter, was welded. The cover fitted onto this end and consisted of two concentric steel discs welded together with a hole through their common center for the pipe. The larger disc was of the same diameter as the cylinder, and the smaller disc was of a diameter slightly smaller than the inner diameter of the annulus. The cover was also fitted with two pieces of half inch pipe, $1\frac{1}{2}$ inches long, each fitted with a stop-cock. The upper end of the center pipe was threaded and extended a short distance above the cover so that the latter could be securely fastened to the cylinder with a washer, gasket, and a metal stop-cock which also served as a nut. The surface of the cover and that of the annulus were carefully finished and a lightly greased fibroid gasket prevented leakage when the cover was bolted on. See Fig. 1.

The central pipe, with stop-cock, served as an inlet for steam. One of the other stop-cocks fastened to the cover served as an inlet for gases, the other stop-cock as an outlet for the distillate.

Two such mills were constructed, and were used in comparative experiments with the two opposite sets of leaflets of the compound leaf of *Ailanthus*. The chopped leaves were placed in the cylinders, 100 stain-

less steel balls, $\frac{1}{2}$ to $\frac{7}{8}$ inch in diameter, and 500 ml. of water were added, and the cover securely closed. The cylinders were filled with the inert gas—by first evacuating, and then filling the cylinders with the gas to a slight positive pressure. The cylinders were again evacuated and

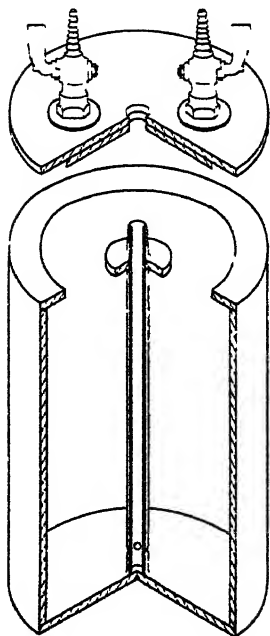


FIG. 1

Apparatus for Grinding and Steam Distilling Leaves in an Inert Atmosphere

The stop-cock which is attached to the central pipe and which fastens the lid, is not shown.

filled another time; this process of evacuation and filling being repeated several times, the cylinders remaining under vacuum for some time, with shaking, in order to free the leaf tissue of air. The cylinders were rotated like pebble mills on rollers at a speed of 36 or 50 r.p.m. For the distillation process connection was made with a steam boiler in such a way that live steam or an inert gas was blown through the connection tubes to free them of all air before the steam was run into the mill. Before starting the steam distillation, connection was made with a distillation apparatus, the inert gas was passed through the mill and into the distillation apparatus until all the air had been replaced. Then steam was admitted into the mill from the boiler and the distillation carried out in a stream of inert gas.

That leaves which are ground in an inert atmosphere and distilled with steam in the apparatus just described, yield little or no condensation product has been demonstrated in 14 separate experiments. In seven of these carbon dioxide was used and no detectable amount of the aldehyde condensation product was obtained. In the other seven experiments nitrogen was used, and in two of these traces of aldehyde were obtained. This may have been due to the fact that the nitrogen contained 0.5 to 1 per cent of oxygen. At any rate the amount of aldehyde obtained from the experiments in which the leaves were ground in air was very much greater than from leaves ground in nitrogen or carbon dioxide, as is illustrated in the following experiment.

A. 0.35 kg. of leaflets from one side of the leaves were chopped and placed in the mill with 500 ml. of water, filled with nitrogen, and ground in this gas for 75 minutes. One liter of hot water was added and the mixture distilled with steam in nitrogen. Yield: none.

B. 0.35 kg. of leaflets from the opposite side of the same leaves used in A. were ground in air in the same manner and steam distilled in nitrogen. Yield: 206 mg. kg.

That leaves which have been ground in an atmosphere of nitrogen and subsequently exposed to oxygen yield hexenal is demonstrated in the following experiment:

0.6 kg. of leaflets from one side of the leaves were chopped and placed in the mill with 500 ml. of water, filled with nitrogen and ground in this gas for 75 minutes. The mill was then evacuated and filled with oxygen, and the leaves ground further in this gas for five minutes. One liter of hot water was added and the mixture distilled with steam in nitrogen. Yield: 130 mg., kg.

The formation of hexenal which takes place on exposing the ground leaves to oxygen can be inhibited by treating the ground leaves with hot water prior to exposing them to oxygen. This is shown in the following experiment:

A. 0.6 kg. of leaflets from one side of the leaves were chopped and placed in the mill with 500 ml. of water and ground in an atmosphere of nitrogen for 75 minutes. One liter of *cold* water was added and the mixture ground for 30 minutes in air. Distillation was carried out in an atmosphere of nitrogen. Yield: 283 mg., kg.

B. 0.6 kg. of leaflets from the opposite side of the leaves were treated in the same manner as in A. with the exception that one liter of *boiling* water was added prior to grinding the mixture with air. It was distilled in an inert atmosphere. Yield: 15 mg./kg.

6. *How is Hexenal Formed?*

It has been shown that hexenal was obtained from ground leaves only when these were exposed to oxygen and that this action is inhibited by heat. These facts might be interpreted to indicate that an oxidative enzyme system of some sort is involved in the formation of hexenal. It would obviously be of much interest to determine the nature of the precursor of the aldehyde and the mechanism of the reactions involved in its formation. The experience gained in recent years with enzymatic systems of leaves and in the field of plant metabolism in general, all serve to emphasize the necessity for caution in speculating on the mode

of formation of any compound found in plants. The systems which are involved in these reactions are of such a complex nature that an enormous amount of exploratory work is necessary before anything approaching a rational concept of plant metabolism can be formulated.

A possibility of a precursor of hexenal is presented by the fact that 3-hexen-1-ol has been found as a component of a wide variety of plant species. The difference in the location of the double bond in the two compounds may not be a serious objection, since it has been found that 3-hexen-1-ol is oxidized to 2-hexenal with chromium trioxide (6). The most direct approach to the proof of the hypothesis that hexenol is the precursor of hexenal in leaves would be experiments designed to determine whether the hexenol content of leaves decreases as the hexenal content increases. But there is no reliable method for the estimation of small quantities of a particular alcohol in aqueous distillates such as exists for the estimation of the aldehyde. The method used by Takei (6) for the estimation of the hexenol and hexenal content of leaves involves the use of very large quantities of leaf material, in no case less than 32 kg. Unfortunately it was not feasible for us to construct the apparatus and to develop the technique for the handling of such large quantities of leaf material which in itself would have been difficult to obtain in a reasonably uniform condition.

Another possible precursor of the hexenal is an unsaturated fatty acid or fat which could be oxidized at one of its double bonds to give the aldehyde. Experiments to test this possibility were carried out with oleic acid. These experiments were carried out in such a manner that oleic acid was added to the ground leaflets from one side of the *Ailanthus* leaves, and the ground leaflets from the other side of the same leaves, without the addition of oleic acid, were used as a control. An increased yield of hexenal was obtained from the ground leaves to which oleic acid had been added. The condensation product consisted essentially of hexenal-*m*-nitrobenzhydrazide, as the product obtained from both sets of leaflets had the same melting point within one or two degrees. This effect was not confined to *Ailanthus*; similar increased yields were obtained when oleic acid was added to the ground leaf material from *Ricinus communis* and *Erodium moschatum*, while *Hedera helix* which yielded only traces of hexenal without oleic acid, yielded no detectable increased amount when this acid was added to the ground leaves. The results are shown in Table I.

The influence of oleic acid on the formation of hexenal may, however, be of a complex nature. Since it was found that this unsaturated acid gave a positive test for peroxide, it is possible that the increased yield of hexenal may have been due to the action of the peroxide. The addition of hydrogen peroxide, although most of it must have been destroyed by the catalase of the leaves, also increased the yield of hexenal. Similarly benzoyl peroxide, though quite insoluble in water, increased the yield. The action of peroxides is, however, in need of more thorough investigation.

In this connection it is not without interest that an antioxidant, such as pyrogallol, definitely inhibits the formation of the hexenal. Thus

TABLE I
Effect of the Addition of Oleic Acid on the Yield of Hexenal

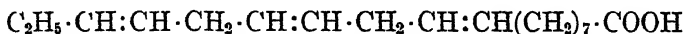
Species	Control Hexenal Condensation Product mg./kg.	5 ml. Oleic Acid Added Hexenal Condensation Product mg./kg.
<i>Ailanthus glandulosa</i>	115	330 (stood 1 hour before distilling)
<i>Ailanthus glandulosa</i>	147	258 (distilled immediately)
<i>Ailanthus glandulosa</i>	160	192 (distilled immediately)
<i>Ricinus communis</i>	180	275 (distilled immediately)
<i>Ricinus communis</i>	146	197 (distilled immediately)
<i>Erodium moschatum</i>	102	139 (distilled immediately)
<i>Hedera helix</i> . . .	Trace	Trace (distilled immediately)

one set of leaflets which was ground in nitrogen for 75 minutes and then ground further in oxygen with the addition of 500 ml. of a 0.3 per cent solution of pyrogallol solution for 30 minutes and steam distilled yielded 104 mg. condensation product per kg. of leaves. The other set of leaves, identically treated with the exception that the pyrogallol solution was added after grinding in oxygen, yielded 218 mg. condensation product on the same basis. The addition of manganous chloride, which specifically enhances peroxidase activity, was found to have no effect on the yield of aldehyde.

The action of the oxidative enzyme system which presumably is responsible for the formation of the hexenal may be analogous to the systems described by Strain (11, 12), which oxidize some fats and carotenoid pigments.

Finally, it may be mentioned that linolenic acid might also be con-

sidered in the formation of hexenal, the latter being formed by oxidative cleavage at the double bond between the twelfth and thirteenth carbon atoms, followed by a shift of the double bond:



Linolenic acid is of particular interest in this connection because of the many plant products which may be derived from it by oxidative fission at one of its double bonds, and, in the case of products containing a carbonyl or carboxyl group, the shift of one of the remaining double bonds to a position in conjugation with those groups. Thus, by cleavage at the double bond between the ninth and tenth carbon atoms, nonadienal, isolated by Ruzicka and Schinz (13) from violet leaves and by Takei from cucumbers, may be obtained. Fission at the twelfth carbon atom gives hexenol and hexenal as products of the hydrocarbon end of the chain, while traumatic acid of English, Bonner and Haagen-Smit (14) would be the final oxidation product of the other end of the chain.

SUMMARY

The evidence which has been presented clearly indicates that the amount of hexenal obtained is largely dependent on the treatment to which the leaves are subjected for the isolation of the aldehyde. The highest yields (without the addition of other substances than water) were obtained from finely ground leaves which had been exposed to oxygen during or after the grinding process and prior to their distillation with steam. Little or no aldehyde is obtained if the leaves are not ground, or if the leaves are not exposed to oxygen during or after the grinding process. Leaves which have been ground in an inert atmosphere, then treated with hot water and exposed to oxygen before distillation, give very much less aldehyde than leaves which are identically ground but treated with cold water, exposed to oxygen and distilled. These facts make it highly probable that the hexenal is formed by an enzymatic process when the ruptured cells of the leaves are exposed to oxygen.

The nature of the enzyme system has as yet not been determined. Pyrogallol, in concentrations as low as 0.1 per cent inhibits the formation of the aldehyde. Oleic acid, containing peroxide, and other peroxides increase the yield of hexenal. The possible rôle of unsaturated acids, such as linolenic acid, in the formation of hexenal is discussed.

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The Rôle of Cephalin and "Thromboplastin" in the Coagulation of Vitamin-K-Deficient Chick Plasma

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INTRODUCTION

The delay in the spontaneous coagulation of the blood of chickens fed a diet deficient in vitamin K is generally attributed to a deficiency in "prothrombin" in the circulating blood or plasma. The experimental basis for this conclusion rests primarily upon the observations of Dam and Schønheyder (1, 2). They observed that fresh juice or saline extracts of breast muscle from either normal or deficient chicks accelerated the clotting of normal and deficient chick plasma; however, the plasma of the normal birds was clotted in from one and one-half to three minutes, whereas that of the vitamin-K-deficient chicks required from seven to fifteen minutes. From these data it was concluded that the clotting activities of the tissues were not affected by the deficiency in vitamin K but that the delay in the clotting of the plasma was due to a lowering in the level of prothrombin in the blood. In confirmation of this view, they subsequently applied methods of prothrombin separation described by Mellanby and by Howell and found that the resulting preparations obtained from deficient chick plasma failed to show prothrombin activity whereas those obtained from normal chick plasma were active (3). This interpretation of the results is derived from and contributes to an oversimplification of theory according to which fresh tissue contains a heat labile constituent designated as "thromboplastin" which transforms prothrombin into "thrombin" or facilitates this transformation by calcium. The thrombin acting on the soluble but inert fibrinogen converts it into the insoluble fibrin. The velocity of clotting is considered to be directly related to the concentration in thrombin. Thus delay in clotting observed in plasma treated with thromboplastin is considered

as due to a deficiency in prothrombin. This view has been widely accepted not only with regard to the delayed clotting of the blood of vitamin-K-deficient chicks but to that observed in a variety of conditions in animals and man (4).

The studies of Bordet and Delange (5) and those from this laboratory (6) indicate that the heat stable lipid fraction of the tissues or platelets or the purified cephalin obtained from them plays a distinctive rôle in the coagulation of the blood as contrasted with those substances classified as thromboplastins. It seemed of interest therefore to investigate further the coagulative behavior of the plasma of chicks deficient in vitamin K particularly with respect to the clotting activities of cephalin as contrasted with the saline extracts of muscle tissues. The breast muscle of exsanguinated chicks seemed particularly suitable for comparative study since it can be secured relatively free from blood constituents and probably contains much less cephalin than the brain or lung tissue usually employed as thromboplastin.

EXPERIMENTAL

Single-comb White Leghorn chicks were used. The basic diet was that described by Almquist and Stokstad (7) in which ether-extracted vitamin-free Labco Casein was substituted for fishmeal. For the experiments recorded in this and the succeeding paper (8) 5 groups of 40 chicks each were used. In each experiment 20 chicks were maintained on the vitamin-K-deficient diet and 20 on the control diet made up with the same proportion of the various constituents in the experimental diet, except that the casein and yeast were not extracted with ether and 2.5 per cent of alfalfa meal was added.

Technic

The blood was collected from the carotid artery with a paraffined canula into chilled paraffined tubes and the plasma separated at high speed in a refrigerated centrifuge. The same technic was used in securing blood for serum except that clean glass tubes were substituted for the paraffined ones and the blood allowed to clot without removal of the cells. The cephalin used was prepared as previously described (9) and possessed a constant and uniform activity. Solutions of cephalin and calcium chloride were made up in saline. Extracts of fresh tissue were prepared by grinding with sand equal quantities of saline and breast muscle from exsanguinated chicks and extracting at 3°-6°C. for twenty-four hours. These extracts were used fresh, or, after separation in the centrifuge, were lyophilized. Clotting experiments were carried out in 11-by-75-mm. tubes. The reagents were diluted so that the amount required in the test was contained in

0.1 ml. Saline was added to bring the volume to 0.6 ml. and the mixture placed in the water bath at 37°C. for from six to ten minutes, when 0.1 ml. of the plasma was added and the time required for clot formation observed.

As a standard of comparison for determining prothrombin activity of serum, the dioxalated plasma described by Bordet and Delange (10) was used. This was prepared by adding four volumes of 0.2-per-cent sodium oxalate, made isotonic with saline, to one volume of cell-free horse plasma oxalated at 0.1 per cent. When fresh, dioxalated plasma gives uniform results in determinations of thrombin activity; it fails to clot on the addition of amounts of cephalin and CaCl_2 that, after incubation with active serum, coagulate it promptly. When weakly oxalated plasma is used or too great an amount of calcium salt is added in the activation of serum (prothrombin), clotting frequently becomes so rapid as to be of little value in differentiating the effect due to serum from the direct effect of the reagents on the plasma. This difficulty also arises when fibrinogen solutions are used to detect thrombin activity. Such solutions are difficult to standardize and either clot spontaneously with time or become permanently incoagulable.

RESULTS

When chicks, one day to two weeks old, were fed the deficient diet they developed not only the characteristic symptoms but many not associated with vitamin-K-deficiency and usually died in from four to five weeks. When 4- to 6-week old chicks were used, the characteristic hemorrhagic symptoms appeared after two to three weeks but the birds were otherwise well nourished and maintained normal growth even when kept for several months on the deficient diet. There occurred, however, a striking depigmentation of the leg scales, skin, comb, beak, and particularly the plasma. Even the yolks of eggs from pullets maintained on this diet were practically colorless. The control chicks on the same diet plus alfalfa showed relatively slight depigmentation. In view of the possibility that the level of vitamin A or carotene in the diet might be too low the following experiment was made.

Doses of 1 mg. of carotene, alone, or with 1 microgram of 2-methyl-1,4-naphthoquinone were administered directly into the crop of a group of depigmented chicks that had been on the vitamin-K-deficient diet for four weeks. The material was given daily over an additional period of four weeks but failed to restore pigment in any of the birds. The coagulative properties of the plasma of the chicks receiving the 2-methyl-

1,4-napthoquinone returned to normal. Thus, although the cause of the depigmentation is not evident, it appears not to be related to altered coagulative properties of the plasma.¹

When the paraffin technic described above was used, the cell-free plasma of chicks bled after four weeks on the deficient diet remained fluid indefinitely either when kept in the paraffined tubes or when transferred to clean glass tubes. The plasma of the normal controls remained fluid for several days when kept in paraffined tubes but after contact with glass clotted within an hour or two. Thus, there is an essential difference in the sensitivity of the two types of plasma in their reaction to contact with a wettable surface. This effect of contact on the clotting of normal cell-free chick plasma cannot be adequately controlled, so that the velocity of clotting observed in subsequent experiments is associated in part with changes resulting from contact with the pipettes and tubes used in the tests. Tests to determine the activity of cephalin, tissue extract, etc., were carried out as soon as possible after collection of the plasma.

The clotting action of cephalin and calcium chloride was tested first. The addition of varying proportions of these reagents failed to clot the plasma from the vitamin-deficient chicks. Clotting of the normal control plasma resulted after from nine minutes to several hours depending in part on the relative proportions of cephalin and calcium chloride and probably in part on individual variations in the stability of the plasma.

Significantly different results were obtained when the saline extract of chick breast muscle was used instead of cephalin. With this reagent, plasma from the vitamin-K-deficient chicks clotted in from five to twenty minutes, whereas that from the normal controls required from two to nine minutes. In confirmation of the results of Schönheyder, no differences were noted in the behavior of the saline extracts of breast muscle from vitamin-K-deficient or normal chicks. Thus the effect of the saline extract of breast muscle on the velocity of clotting was very marked whereas cephalin failed completely to clot deficient chick plasma and in some instances was almost without effect on the normal plasma. The essential comparisons of one experiment are given in Table I.

In view of the difference in the behavior of cephalin and the saline extract of muscle tissue in the presence of calcium chloride, experiments

¹ Analyses of the weights of chicks fed adequate and deficient diets and of the inorganic and lipid phosphorus and calcium in the cell-free plasma of the normal and deficient chicks are published separately (8).

were made to ascertain what their effect would be when mixed with fresh serum and incubated briefly at 37°C. before the addition of the plasma. According to the thrombin hypothesis, this technic should reveal the relative capacity of the reagents to engender thrombin from the prothrombin that is present in fresh normal serum but is considered to be diminished or absent from the sera of animals or birds deficient in vitamin K. Fresh sera from the normal chicken, guinea-pig, rabbit, and

TABLE I

Comparison of the Clotting Activity of Cephalin and of a Saline Extract of Chick Breast Muscle for Plasma of the Vitamin-K-Deficient Chick

Reagents	Quantities in ml.									
Cephalin, 0.01 per cent....	0.1	0.1			0.1	0.1				
Saline extract of chick breast muscle 1:10... ..			0.1	0.1			0.1	0.1		
Saline... ..	0.4	0.4	0.4	0.4	0.5	0.5	0.5	0.5	0.5	0.5
Calcium chloride, N/100 .	0.1	0.1	0.1	0.1					0.1	0.1
In water bath for 6 minutes at 37°C... ..										
Vitamin-K-deficient chick plasma	0.1	0	0.1	0	0.1	0	0.1	0	0.1	0
Normal chick plasma.....	0	0.1	0	0.1	0	0.1	0	0.1	0	0.1
Clotting time during 3-5 hours.....	No clot	27 min.	6 min.	2 min.	No clot	No clot	21 min.	5 min.	No clot	3 hrs. 23 min.

horse were used after allowing sufficient time for the dissipation of any so-called thrombin activity that they might possess. Serum from the deficient chick was not used since with the technic described the blood failed to clot.

The deficient chick plasma was clotted by the incubated mixtures of cephalin, calcium chloride, and active serum from the guinea-pig, rabbit, and horse in from one to eight minutes. Active normal chick sera, however, under the same conditions, required from one to several hours, thus indicating a significant difference in the clotting behavior of such

sera. When the breast-muscle extract was used instead of cephalin under the same conditions with the active sera and calcium chloride the deficient and the control chick plasma were clotted, but little or no change in the time required was observed as compared with that required with breast-muscle extract and calcium chloride without serum. One must conclude from these results that whereas the saline extracts of chick breast muscle possess *per se* a marked clotting activity for deficient

TABLE II

Comparison of the Clotting Activity of Fresh Serum with Cephalin or with Saline Extract of Chick Breast Muscle for Plasma of the Vitamin-K-Deficient Chick

Reagents	Quantities in ml.											
Fresh guinea-pig serum, 1:10	0.1	0.1										
Fresh normal chick serum, 1:10			0.1	0.1					0.1	0.1	0.1	0.1
Cephalin, 0.01 per cent	0.1		0.1				0.1			0.1		
Saline extract of chick breast muscle, 1:10		0.1		0.1					0.1			0.1
Saline	0.3	0.3	0.3	0.3	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4
Calcium chloride, N 100.	0.1	0.1	0.1	0.1			0.1				0.1	
In water bath for 6 minutes at 37°C												
Vitamin-K-deficient chick plasma	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Clotting time during 3-5 hours	4	6	3	1*	No	13	5½	12	No	No	No	6
	min.	min.	hrs	min.	clot	min.	min.	min	clot	clot	clot	min.

* This result suggests an activation since the clotting time is only 1/6 that of the control; in similar additional experiments however this relationship was approximately 1:2.

For controls of cephalin or tissue extract with calcium chloride, see Table I

as well as normal chick plasma they have little or no effect in activating the sera from the guinea-pig, rabbit, horse, or chicken. Tables II and III illustrate the results.

Since the consensus of data in the literature indicates that the prothrombin activity of serum is heat labile, being similar in this respect to complement activity, the experiments were repeated after heating the sera at 56°C. for one hour. The mixtures containing cephalin and calcium chloride with heated guinea-pig or rabbit serum clotted the plasma from deficient chicks in about twenty minutes on the average, thus indicating a marked activation although not so great as when fresh sera were used. With the mixtures containing heated horse serum, clotting required about an hour, whereas those containing heated chick serum failed to clot. With normal chick plasma the heated guinea-pig and rabbit sera showed only a slightly increased activity and the heated horse or chicken serum did not modify the velocity of clotting observed

with cephalin and calcium chloride alone. The heated, like the active, sera had no significant effect on the clotting time of the plasma of deficient or control chicks after preliminary incubation with the saline extract of breast muscle and calcium chloride without serum. The most significant result of these tests is that, after being heated at 56°C. for one hour, a procedure which presumably destroys prothrombin activity, certain mammalian sera have the capacity of restoring in conjunction

TABLE III

Comparison of the Clotting Activity of Fresh Serum with Cephalin or with Saline Extract of Chick Breast Muscle for Plasma of the Normal Chick

Reagents	Quantities in ml.											
Fresh guinea-pig serum, 1:10	0.1	0.1			0.1	0.1	0.1	0.1				
Fresh normal chick serum, 1:10.			0.1	0.1					0.1	0.1	0.1	0.1
Cephalin, 0.01 percent	0.1		0.1			0.1				0.1		
Saline extract of chick breast muscle, 1:10		0.1		0.1				0.1				0.1
Saline	0.3	0.3	0.3	0.3	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4
Calcium chloride, N/100	0.1	0.1	0.1	0.1			0.1				0.1	
In water bath for 6 minutes at 37°C.												
Normal chick plasma	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Clotting time during 3-5 hours	2	2	29	1	1	9	39	3	No	1 hr.	1 hr.	2
	min.	min.	min.	min.	hr.	min.	min.	min.	clot	20 min.	48 min.	min

For controls of cephalin or tissue extract with calcium chloride, see Table I

with cephalin and calcium chloride the coagulability of the plasma of vitamin-K-deficient chicks. See Tables IV and V.

Further experiments were made using the dioxalated plasma of Bordet and Delange as a reagent to test for thrombin formation in incubated mixtures of fresh sera with CaCl_2 and saline extracts of chick breast muscle or of heated sera with cephalin and calcium chloride. While control mixtures of all of the fresh mammalian sera with cephalin and calcium chloride clotted the dioxalated plasma promptly, fresh normal chick serum under the same conditions failed to do so. Mixtures of the heated sera with CaCl_2 and cephalin were totally inactive as were also the mixtures of breast-muscle extract and CaCl_2 with either fresh or heated sera from either mammal or chick. Table VI.

From the experiments described, certain differences become evident in the clotting activities of chick and mammalian plasma and serum. Thus the fresh sera of the guinea-pig, horse, or rabbit appear to contain prothrombin as indicated by the clotting activity that develops in in-

TABLE IV

Comparison of the Clotting Activity of Heated Serum with Cephalin or with Saline Extract of Chick Breast Muscle for Plasma of the Vitamin-K-Deficient Chick

Reagents	Quantities in ml.											
Guinea-pig serum heated for 1 hour at 56°C., 1:10	0.1	0.1			0.1	0.1	0.1	0.1				
Normal chick serum heated for 1 hour at 56°C., 1:10			0.1	0.1					0.1	0.1	0.1	0.1
Cephalin, 0.01 per cent . .	0.1		0.1				0.1			0.1		
Saline extract of chick breast muscle, 1:10		0.1		0.1					0.1			0.1
Saline	0.3	0.3	0.3	0.3	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4
Calcium chloride, N, 100 . .	0.1	0.1	0.1	0.1			0.1				0.1	
In water bath for 6 minutes at 37°C.												
Vitamin-K-deficient chick plasma	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Clotting time during 3-5 hours	11	6	No	4	No	32	No	17	No	No	No	11
	min.	min.	clot	min.	clot	min.	clot	min.	clot	clot	clot	min.

For controls of cephalin or tissue extract with calcium chloride, see Table I.

TABLE V

Comparison of the Clotting Activity of Heated Serum with Cephalin or with Saline Extract of Chick Breast Muscle for Plasma of the Normal Chick

Reagents	Quantities in ml.											
Guinea-pig serum heated for 60 minutes at 56°C., 1:10.....	0.1	0.1			0.1	0.1	0.1	0.1				
Normal chick serum heated for 60 minutes at 56°C., 1:10			0.1	0.1					0.1	0.1	0.1	0.1
Cephalin, 0.01 per cent	0.1		0.1			0.1				0.1		
Saline extract of chick breast muscle, 1:10		0.1		0.1				0.1				0.1
Saline	0.3	0.3	0.3	0.3	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4
Calcium chloride, N, 100 . .	0.1	0.1	0.1	0.1			0.1				0.1	
In water bath for six minutes at 37°C.												
Normal chick plasma	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Clotting time during 3-5 hours . .	10	2	30	1	No	1 hr.	1 hr.	2	No	1 hr.	2 hr.	2
	min	min.	min.	min.	clot	39 min.	43 min.	min.	clot	44 min.	21 min.	min.

For controls of cephalin or tissue extract with calcium chloride, see Table I.

cubated mixtures of these sera with cephalin and CaCl_2 for both chick and dioxalated horse plasma; on the basis of similar experiments, fresh chicken serum appears not to contain prothrombin. Chick-muscle

extract fails to activate any of the sera to bring about clotting of dioxalated horse plasma although in the experiment with normal and vitamin-K-deficient chick plasma it was a more effective clotting agent

TABLE VI

Comparison of the Clotting Activity of Fresh and Heated Serum with Cephalin or with Saline Extract of Chick Breast Muscle for Dioxalated Horse Plasma
Reagents Quantities in ml.

Fresh guinea-pig serum, 1:10.	0.1											0.1
Fresh normal chick serum, 1:10.....		0.1										0.1
Guinea-pig serum heated for one hour at 56°C., 1:10...			0.1		0.1							
Normal chick serum heated for one hour at 56°C., 1:10 ..					0.1		0.1					
Cephalin, 0.01 per cent. . .							0.1	0.1	0.1	0.1		0.1
Saline extract of chick heart muscle, 1:10 .	0.1	0.1	0.1	0.1								0.1
Saline....	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4	
Calcium chloride, N/100...	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
In water bath for six minutes at 37°C.....												
Dioxalated horse plasma... .	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
Clotting time during 3-5 hours.....	No clot	No clot	No clot	No clot	No clot	No clot	2 min.	No clot	No clot	No clot		

Control tests of each serum alone or with cephalin, calcium chloride or muscle extract, as well as of muscle extract or calcium chloride alone failed to clot.

than cephalin, bringing about almost as rapid coagulation in the absence as in the presence of fresh serum. Heat inactivated mammalian sera, together with cephalin and calcium chloride, clotted deficient chick

plasma quite rapidly although in tests with dioxalated horse plasma none of the heated sera showed evidence of prothrombin activity when incubated with calcium chloride and either cephalin or muscle extract.

Obviously more work must be done before a satisfactory interpretation of these results can be made but it seems clear that the delay in the clotting of the blood of chicks fed a diet deficient in vitamin K is not adequately explained as due to a deficiency in prothrombin.

SUMMARY

Chicks placed on a vitamin-K-deficient diet when 4 to 6 weeks old maintained normal growth but developed characteristic hemorrhagic symptoms and, moreover, failed to show the yellow pigment in tissues and blood regularly observed in normal chicks. Administration of carotene and 2-methyl-1,4-napthoquinone did not restore pigment although the coagulability of the blood and plasma returned to normal.

The cell-free plasma obtained from the vitamin-K-deficient chicks by the paraffin technic remained fluid indefinitely whether stored in paraffined or clean glass containers; that of the normal controls showed a marked delay in clotting when kept in paraffined tubes but clotted usually within an hour or two after transfer to clean glass tubes.

The blood plasma of vitamin-K-deficient chicks failed to clot after incubation with cephalin and calcium chloride but clotted promptly when fresh or heat-inactivated serum from mammals was added. The cell-free plasma of the normal controls was clotted by cephalin and calcium chloride after some delay; results were irregular due probably to the difficulty of controlling the effect of contact with a wettable surface. Active mammalian serum, together with cephalin and calcium chloride clotted control chick plasma promptly; the heated serum, only slightly more rapidly than calcium chloride and cephalin alone. Fresh normal chick serum, after incubation with cephalin and calcium chloride had little or no activity in the clotting of plasma either from normal or deficient chicks.

No difference in the coagulative activity was detected in the saline extracts of breast muscle from normal and from deficient chicks. In the presence of calcium chloride they clotted normal chick plasma promptly—within two to three minutes—and deficient chick plasma within five to fifteen minutes. Neither fresh nor heated serum, from mammal or chick, when incubated with muscle extract and calcium

chloride, showed any appreciable acceleration of clotting over that observed with extract and calcium chloride alone.

A comparison of the prothrombin activity of the fresh and heated sera from mammals and chicks, by preliminary incubation with calcium chloride and cephalin before addition of dioxalated horse plasma as reagent of thrombin formation, demonstrated marked prothrombin activity in the fresh but not in the heated mammalian sera or in either the fresh or heated serum from the normal chick. When, in similar experiments, breast-muscle extract was used instead of cephalin, dioxalated plasma was not clotted in any instance, suggesting, as do the experiments with chick plasma, a distinct difference between the rôle of such extracts and that of cephalin in clotting.

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Chemical Analyses of the Blood Plasma of Chicks Deficient in Vitamin K

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INTRODUCTION

During a study of the coagulative behavior of the blood plasma of chicks fed a diet deficient in vitamin K, analyses were made of the inorganic and total lipid phosphorus as well as of the calcium content of the plasma. This was undertaken since, as previously observed with mammalian plasma (1), a small quantity of trisodium phosphate when added to plasma caused an inhibition of clotting similar to that observed with the plasma of chicks deficient in vitamin K. Furthermore, Schönheyder (2) had noted that the serum from chicks deficient in vitamin K had more inorganic phosphorus and less calcium than that from normal chicks. Schönheyder's studies were made on very young chicks which not only show the characteristic hemorrhagic symptoms associated with a deficiency in vitamin K but also other symptoms, particularly leg weakness, failure to maintain normal growth, and delay in, or faulty development of feathers. These birds usually die after four to five weeks on the vitamin-deficient diet.

EXPERIMENTAL

In our studies (3) it was noted that these symptoms of general malnutrition did not appear when baby chicks were maintained on an adequate diet for from four to six weeks before being placed on the diet deficient in vitamin K. The birds remained normal in appearance even after several months on the deficient diet. The hemorrhagic symptoms developed as quickly and regularly as in the younger chicks and some evidence of gizzard erosion was usually present. In addition, with the diet used, there was a failure to develop the characteristic yellow pig-

ment throughout the tissues and blood observed in chicks on the control diet, which is similar to the deficient diet with the exception of the addition of 2.5 per cent of alfalfa meal and the use of yeast and casein which were not ether extracted. The coagulative behavior of the blood plasma, however, as previously noted, appears not affected by the presence or absence of the pigment.

The present report deals with the results obtained when two groups of day-old White Leghorn chicks were maintained on an adequate control diet for twenty-four days, after which vitamin K was eliminated from the diet of one of the groups, there being twenty chicks in each. Equal numbers from each group were studied at the end of given periods thereafter as follows: 29, 31, 33, 36, 38, 40, and 43 days—three from each group at each time but the last, when the remaining two of each were taken. All the chicks were weighed when the one group was placed on the deficient diet and at the end of the experimental period. Then they were bled from the carotid with a paraffined canula into chilled paraffined tubes containing one part of heparin to 1000 parts of blood and the cells separated immediately by centrifuging at 2°C. A portion of the blood from each chick was collected in paraffined tubes without heparin. The clotting qualities of the plasma collected without heparin were studied and found to show the same differences previously noted (3) with the cell-free plasma from similar groups of vitamin-K deficient and control chicks.

Inorganic phosphorus (4) and calcium (5) were estimated by colorimetric methods, lipid phosphorus by a microgravimetric procedure (6). The inorganic phosphorus was also estimated by gasometric analysis (7).¹

The results are presented graphically using a simple method based on a system of statistical analysis developed elsewhere (8, 9). The data were treated as sets of random observations under the given conditions, neglecting variations in the experimental interval. Cumulative probability graphs were constructed based essentially on the following relations.

With regard to a given variate (x), the character quantitatively determined, let n random observations be arranged in ascending order of magnitude. Then

¹ In the gasometric estimation of inorganic phosphorus the carbon phosphorus ratio for the precipitate, as determined with our reagents, appeared somewhat higher than that reported by Hoagland and previously by Kirk. Our value was about 33.4, equivalent to approximately 4 molecules of strychnine to 1 atom of phosphorus.

the probability is exactly $1/(n + 1)$ that any future observation (x) lie below all these and the probability is the same that it lie between any successive two in the original set. Thus, the probability is $P = k/(n + 1)$ that this value of x is below the k th of the original set in ascending order of magnitude. A plot of P against the corresponding observed value of x gives the cumulative probability graph. The points are joined to aid the eye. In this way all the results under each set of circumstances are presented in a simple graph for comparison with other related data similarly presented.

RESULTS

In Fig. 1 are given the weight graphs for each group of twenty chicks at the beginning of the experimental interval. These are in close agree-

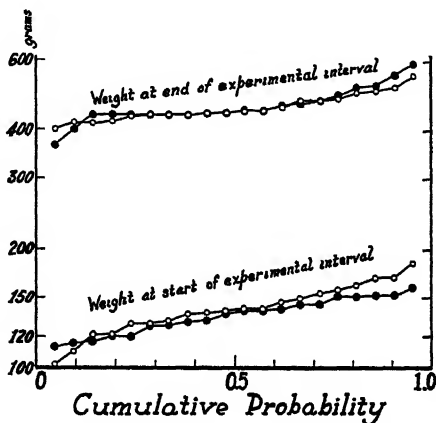


FIG. 1

Large full circles represent chicks on normal diet; open circles represent those on vitamin-K-deficient diet during the experimental interval.

ment, as they should be, each group having had similar treatment up to this point. Likewise, in the same figure are given weight graphs for the end of the experimental period. The similarity of weight distribution regardless of vitamin-K-deficiency is striking and in harmony with the normal appearance of the birds, but in marked contrast to the retardation of growth and general malnutrition resulting when day-old chicks are placed on the deficient diet.

The results of analyses of the plasma by the colorimetric and gravimetric methods at the end of the experimental period are presented in the same manner in Fig. 2. The vitamin-K-deficient and control chicks

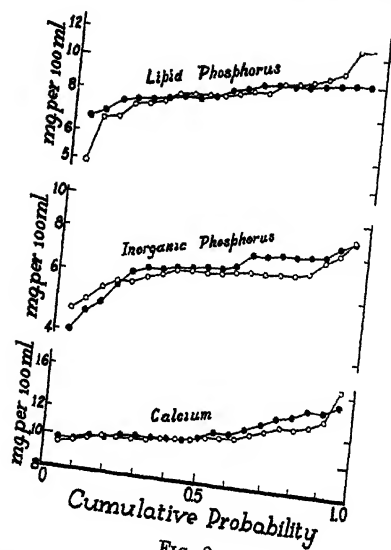


FIG. 2

Results of plasma analysis for normal chicks represented by large full circles, those for vitamin-K-deficient chicks by open circles.

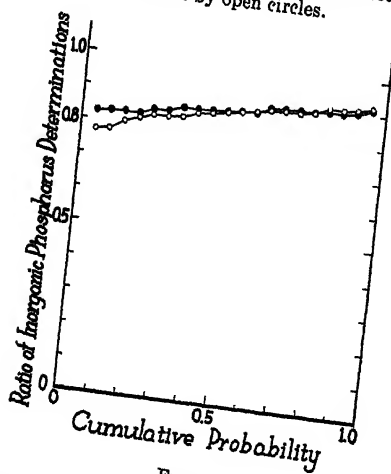


FIG. 3

Ratio = colorimetric value: gasometric value on same plasma. Large full circles for normal, open circles for deficient chicks.

exhibit a close similarity with regard to inorganic phosphorus, total lipid phosphorus, and calcium.

In the gasometric estimation of inorganic phosphorus, it was noted that the results were uniformly higher than with the colorimetric estimations. A comparison was made between the estimations made by the colorimetric and gasometric methods on the same plasma designated respectively as P_c and P_g . For the comparison, x was taken as the ratio, $P_c:P_g$. The graphs are given in Fig. 3. It is interesting that a difference (P_c less than P_g) appears throughout the two sets of twenty plasmas and, moreover, that the distribution of the ratio ($P_c:P_g$) is nearly the same in each set regardless of the gross differences in the color and clotting qualities.

Neither the inhibition of clotting nor the absence of pigment observed in chicks fed the modified Almquist diet (3) can be related to quantitative changes in the inorganic or lipid phosphorus or the calcium of the plasma. An investigation is underway to determine if qualitative differences, particularly of the phosphatides, may have some significance. The differences in inorganic phosphorus noted by Schønheyder were probably associated with the marked differences in nutritional state observed in studies with younger chicks.

SUMMARY

Two groups of baby chicks were fed an adequate diet for twenty-four days; then for a period of four to six weeks vitamin K was excluded from the diet of one group. The chicks were weighed at the beginning and end of the experimental period when the cell-free plasma was analyzed for inorganic and lipid phosphorus and calcium. A statistical analysis of the observations by the method of cumulative probability indicated no significant differences in the weight of the two groups nor in the quantitative estimates of inorganic and lipid phosphorus or calcium.

The authors are indebted to Mr. H. W. Eckert for most of the chemical analyses.

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The Storage and Interaction of Water Soluble Vitamins in the Malpighian System of *Periplaneta Americana* (L.)

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INTRODUCTION

The malpighian system of insects is generally considered to function solely as an organ of excretion. It is the purpose of this paper to present evidence that this system also functions as a storage organ for various vitamins in a manner analogous to the mammalian liver and to describe the action of certain of the B-complex vitamins upon the metabolism of riboflavin.

Many investigators have been puzzled by the nature of the yellowish pigments so frequently encountered in the malpighian tubes of insects. Veneziani (1) named the pigment entomo-urochrome, in the belief that it was chemically related to the mammalian bile pigment urochrome. Wigglesworth (2) stated that the pigment in the tubes of *Rhodnius* was distinctly different from this, but offered no suggestions as to its nature. Drilhon and Busnel (3) reported that varying amounts of an unidentified flavin were present in the malpighian tubes of a number of insects. In a previous paper, Metcalf and Patton (4) have shown that the yellow pigment which is a characteristic feature of the malpighian tubes of the American roach, *Periplaneta*, is identical with synthetic riboflavin and have described its dimorphic nature.

EXPERIMENTAL

Methods

Assays for certain of the water soluble vitamins were carried out by extirpating the entire malpighian system from an adult roach, freeing

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the organ from foreign tissues, weighing, and macerating the whole in an aliquot of distilled water, followed by appropriate treatment for the complete liberation of the factor to be assayed. Riboflavin and pantothenic acid were determined microbiologically with *Lactobacillus casei* (5, 6); nicotinic acid with *Lactobacillus arabinosus* (7); thiamin by growth of *Phycomyces blakeleeanus* (8) and ascorbic acid by microtitration with 2,6-dichlorophenolindophenol (9). Samples of the unknowns were run in duplicate at four levels; and results were considered valid only when the values obtained, checked for at least two levels. Each value expressed in the table represents the average of several samples from one insect.

Observations of riboflavin metabolism in living insects were made by means of the "intravital" fluorescence microscope (Ellinger, 10). Essentially this method consists in the illumination of the object from above by a beam of intense ultra-violet radiation which enters the microscope tube through a side arm, and is reflected down through the objective by a small front-surfaced aluminum mirror. The objective focuses the illuminating rays on the object where fluorescence is produced, which is observed through the microscope. The large amounts of brilliantly fluorescent riboflavin present in the roach make this insect very suitable for such a study. The insect was etherized, pinned dorsum downward, and a small window was cut in the ventral body wall. The malpighian system was exposed to view by pushing aside the fat body, and the opening was sealed by coating the edges of the incision with petrolatum and applying a fragment of microscope cover slip to seal in the abdominal contents. While the insect was under observation, physiological saline solutions containing various materials were injected into the blood stream of the insect, and the visual changes in fluorescence of the riboflavin present were observed.

Vitamin Content of the Malpighian Tubes

The very intense fluorescence of riboflavin in the malpighian system of *Periplaneta* made an assay desirable. Preliminary figures showed such high riboflavin concentration that the assays were extended to the other water soluble vitamins to see if comparable concentrations existed. The values obtained for five water soluble vitamins are shown in Table I.

The riboflavin content, about 30 times that of beef liver, probably represents the richest known natural concentration. This is in good agreement with the assay figures reported by Drilhon and Busnel (3)

for a flavin found in the malpighian tubes of several insects, which reached a concentration of 2150 $\mu\text{g. per g.}$ in the grasshopper, *Schistocerca*. The thiamin content is about 10 times that of beef liver, while the contents of pantothenic acid, nicotinic acid, and ascorbic acid are from 1.5 to 2 times those of beef liver.

From these results, it is evident that there is a phenomenal concentration of riboflavin and thiamin, in the malpighian system of this insect. It can be concluded that one of the principal functions of the malpighian tubes, which had been previously unsuspected, is the storage of large quantities of water soluble vitamins in a manner analogous to the vertebrate liver and kidney.

TABLE I

Water Soluble Vitamin Content of Malpighian Tissue of Periplaneta
All Values in Micrograms per Gram of Fresh Tissue

Material	Thiamin	Riboflavin	Nicotinic acid	Panto- thenic acid	Ascorbic acid
<i>Periplaneta</i> 1 . .	42	1,000	200	80	600
2 .	50	840	480		620
3	33				1,012

Dimorphism of Riboflavin

The most striking feature of the malpighian system of *Periplaneta* as revealed by the fluorescence microscope was the presence of two distinct modifications of riboflavin. One type, *free riboflavin*, was present in solution in the lumina of the distal portions of the tubes as a yellow-green fluorescent fluid. It diffused readily from the tubes after death of the insect and flowed from any rupture of the tube wall. It was very readily reduced by the injection of dilute solutions of sodium hydrosulfite to a non-fluorescent "leuco" state and was reoxidized slowly by air or rapidly with dilute hydrogen peroxide. The other type of riboflavin, *cytoplasmic riboflavin*, was found only in the proximal (basal) portions of the tubes, where it occurred as orange-fluorescent grains (0.5 to 1.5 μ in diameter) in the cytoplasm of the cells comprising the tube walls. It was non-diffusible and unaffected by reduction or oxidation. In the middle portions of the tubes, both forms of riboflavin were present.

The malpighian tubes of roaches fed a riboflavin-free diet for three or four weeks contained only the cytoplasmic form. After 3 months on this diet, no trace of either form of riboflavin could be found.

To determine if any excretion of riboflavin occurred through the malpighian tubes, an adult roach was isolated and fresh feces were examined under the fluorescence microscope. These showed only a dull blue fluorescence even when dissolved in alcohol. The application of dilute hydrogen peroxide had no effect on the fluorescence. The same roach was given 0.02 cc. of 0.001 per cent riboflavin solution *per os*. Examination of the feces over a 24 hour period revealed no trace of the yellow-green fluorescence of riboflavin. A relatively large quantity of feces (0.1 g.) was collected from a number of roaches and macerated in 50 per cent methyl alcohol. The undissolved sediment was centrifuged out, and the supernatant liquid chromatographed upon magnesium oxide. No trace of riboflavin appeared in the column although the same procedure was effective in isolating quantities of riboflavin from the malpighian tubes.

The above results indicate that the large concentrations of riboflavin in the malpighian system of *Periplaneta* are present as a storage supply and not as an intermediate in the excretion of this material. Free riboflavin is absorbed from the blood by the distal portions of the tubes and is stored in a "bound" metabolized form in the proximal portions of the tubes. Upon prolonged riboflavin deficiency, the storage form is gradually mobilized. Ellinger (11) has described a similar condition in the kidney cells of frogs and rats. He observed a yellow-green fluorescent diffusible pigment in the lumina of the kidney tubes and a yellowish fluorescent form present as granules in the cytoplasm of the cells. He states that the yellowish form is not a lactoflavin-protein compound as these are non-fluorescing.

Redox Systems of the Malpighian Tubes

The presence of very large quantities of riboflavin, a constituent of numerous oxidation-reduction carriers should profoundly affect the redox potentials of the malpighian system. Using the methods of Cohen (12) with oxidation-reduction indicators, attempts were made to measure redox potentials in various portions of the tubes. Methylene blue and 2,6-dichlorophenolindophenol were both readily decolorized by the riboflavin containing portions of the tubes, but not so readily by the flavin-free distal portions. Indigo disulfonate was reduced to a slight extent, but safranin was not reduced. The rate of reduction of methylene blue was several times greater in the flavin-containing portions than it was in the flavin-free portions. It can be concluded that the redox potentials of the malpighian tubes are variable, depending upon their

riboflavin content. In the flavin-containing areas, the potential appears to lie between -0.125 volts (indigo disulfonate) and -0.289 volts (safranin) probably near the -0.21 volts potential of free riboflavin.

Spectroscopic examination of minced malpighian tissue failed to reveal any trace of the characteristic absorption bands of the cytochromes (Keilin, 13) even when reduction with sodium hydrosulfite was employed, although cytochrome could be detected in the muscle tissue of this insect. The nitroprusside test for glutathione was also negative on malpighian tissue, but was positive on muscle tissue. The addition of *p*-phenylenediamine and α -naphthol solutions to malpighian tissue gave a blue color, indicating the presence of cytochrome oxidase. Monophenol and polyphenol oxidases could not be detected.

It seems difficult to correlate physiologically the very large amounts of riboflavin present, with the minute amounts of oxidative enzymes found. Ball (14) has shown that in a variety of animal tissues, the amounts of cytochrome and flavin containing enzymes are roughly equivalent while the amount of the phosphopyridine nucleotides is many times greater. The disproportion of these enzyme-forming materials in the malpighian tissue would seem to show that the majority of the riboflavin is present mainly as a storage deposit and is relatively inert physiologically.

Studies on the Interrelations of the B-Vitamins

The very large concentration of riboflavin in the malpighian tubes of *Periplaneta* and its easy accessibility to intravital observations, made this an exceptional opportunity for the study of the interrelationships of the B-complex vitamins. All of the obtainable water soluble vitamins were studied *in vivo* by the injection of solutions into roaches being observed by the intravital microscope. Definite physiological effects were observed with riboflavin, nicotinic acid, thiamin, and pantothenic acid, while pyridoxin, ascorbic acid, inositol, and *p*-aminobenzoic acid were ineffective in producing any observable results. A summary of this study is given in Table II.

Riboflavin injected into the blood stream was soon absorbed by the distal portions of the malpighian tubes and was greatly concentrated. No evidence of the formation of the cytoplasmic flavin was obtained.

Nicotinic acid had a profound effect on the state of oxidation as revealed by an immediate increase in the intensity of riboflavin fluorescence. This resembled the results of the application of dilute hydrogen peroxide to the tubes, but occurred at very much greater dilutions.

TABLE II
Interrelationship of B-Vitamins in Malpighian System of Periplaneta

Vitamin	Least concentration producing visible effect	Time		
		30 seconds	2 minutes	15 minutes
Riboflavin	1/1,000,000	No change.	Tubes become faintly yellow-green in distal portions.	Perceptible increase in concentration of yellow-green in lumina. Greatly increased concentration of yellow-green in lumina.
Nicotinic acid	1/10,000,000	Tubes with free flavin brightened tremendously.	Yellow-green has appeared even in tubes that were previously dull blue fluorescent. Yellow-green material diffusing from tubes into blood stream.	No effect as yet on cytoplasmic flavin. No effect on cytoplasmic form. Brightness of free form somewhat diminished.
Thiamin chloride + HCl	1/100,000	Free flavin has become brighter.	No further change.	Cytoplasmic form has become cloudy. Outline of cells, less definite.
d-Calcium pantothenate	1/1,000,000	Slight brightening of free flavin.	Cytoplasmic form rapidly losing definition, being transformed to free flavin.	Cytoplasmic form has lost definite granular appearance, free form has developed. All tubes now contain free flavin.

Tubes in which riboflavin was not previously visible became yellow-green fluorescent in a very short time after the application of nicotinic acid at a dilution of 1/10,000,000. The permeability of the tubes to riboflavin appeared to be altered by the presence of nicotinic acid, as large amounts of riboflavin were observed to diffuse from the tubes whenever nicotinic acid was added. Nicotinic acid had no apparent effect on the cytoplasmic form of flavin. A similar effect was observed in mammalian kidneys by Hirt and Wimmer (15) following the injection of nicotinic acid solution into the blood stream of a mouse. These authors state that within 10 minutes, bright green fluorescent, light labile particles appeared in the star cells and endothelium of the liver. Later a strong yellow fluorescence appeared. They concluded that the fluorescence produced was due to complex nicotine-amide-protein compounds, a conclusion on which the above results would cast some doubt.

Thiamin also had a definite effect on the behavior of riboflavin. Dilutions of 1/100,000 somewhat increased the brightness of the free flavin, but the principal effect was in a slow conversion of the orange-fluorescent cytoplasmic form of flavin into the free yellow-green fluorescent form. This occurred over a period of about 15 minutes, beginning with a slight blurring of the minute fluorescent particles and continuing until they were entirely converted to the free form. Hirt and Wimmer (16) stated that the injection of thiamin into the blood stream of a mouse caused the formation of complex compounds of riboflavin which appeared as yellow to yellow-green drops in the liver and kidneys.

A third B-vitamin having a visible effect on the fluorescent appearance of the malpighian system was pantothenic acid. Dilutions as great as 1/1,000,000 produced only a slight brightening of the free form of riboflavin, but caused a rapid transformation of the cytoplasmic form into the free form. This effect while similar to that produced by thiamin was much more rapid and more pronounced.²

A recent paper by Supplee, *et al.* (17) has a direct bearing upon the above observations. It was observed by these workers, that the riboflavin content of rat livers was greatly affected by pantothenic acid and to a lesser degree by thiamin. These two vitamins apparently had a specific action on the riboflavin mobilizing function, even after prolonged flavin deficiency.

The experiments described above indicate that the cytoplasmic storage

² The apparent discrepancy between the large amounts of vitamins stored in the tubes and the intense physiological activity of injected vitamins can best be explained by the presence of inert chemical linkages of stored vitamins to tissues.

form of riboflavin is reconverted to the free form by pantothenic acid and to some extent by thiamin, and is thus made available for distribution. Nicotinic acid although not affecting the storage form, conceivably serves to release the free flavin from the malpighian system into the blood stream (note the effect on the permeability of the malpighian tubes for riboflavin) where it is distributed throughout the body.

SUMMARY

Very large amounts of water soluble vitamins are stored in the malpighian tubes of the roach, *Periplaneta*. The content in $\mu\text{g.}$ per g. of fresh tissue is: riboflavin 840–1000, thiamin 33–50, nicotinic acid 200–460, pantothenic acid 80, and ascorbic acid 600–1012. The riboflavin is present in two forms, as free riboflavin and as a “bound” cytoplasmic storage form. Only very minute amounts of oxidative enzymes usually associated with riboflavin systems are present in the malpighian tubes. Certain other B-vitamins present in the malpighian tubes, exert important influence upon the riboflavin storage and mobilization. Pantothenic acid is effective in converting the cytoplasmic form of flavin to the free form. Thiamin acts in much the same way but is slower and less effective. Nicotinic acid acts to disperse the free flavin from the malpighian tubes into the blood stream of the insect, but does not affect the cytoplasmic form.

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Detoxication of Borneol by Glucuronic Acid in Humans

II. Pathological Cases*

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INTRODUCTION AND RESULTS

A previous study (1) had as its aim the establishment of normal values for the detoxication of borneol. Using 26 normal subjects, the ingestion of 1 g. of borneol resulted in the urinary excretion within 24 hours, of an average of 81 per cent as glucuronide, the range being from 60–100 per cent.

This exploratory paper reports results on pathological cases following the ingestion of the same quantity of borneol and using the same method of analysis.

Tables I, II, and III show the correlation between the estimated liver damage based on the usual clinical tests, and that determined from detoxication figures. It appears logical to consider a detoxication of between 45–60 per cent as "slight impairment of detoxication," and to regard anything less than 45 per cent as "severe impairment of detoxication." Twenty-eight hospitalized patients were used in this study.

There are 14 cases of severe disease of the liver parenchyma (Table I). These would be expected to show low percentages of urinary glucuronides. Table II gives results on 6 cases which were believed to have mild impairment of liver function. Table III presents the results on 8 cases of various diseases not believed to impair the function of the liver parenchyma. The latter might be expected to show normal elimination of glucuronides.

* Aided by a grant from The Ella Sachs Plotz Foundation.

The diagnosis and the estimates of probable liver function are based on the usual clinical and laboratory tests—including galactose tolerance

TABLE I
Cases of Liver Disease Believed to Have Severe Parenchymatous Damage

Patient No.	Clinical Diagnosis	Per Cent Detoxication	Correlation	Blood Cholesterol mg. 100 cc.	Ester mg. 100 cc.	Icterus Index	Galactose Test g. in 5 hrs.	Urine Urobilinogen Dilution	Blood Phosphatase King-Armstrong Units	Prothrombin Per Cent of Normal	Confirmation of Diagnosis	Remarks
1	Laennec's Cirrhosis with Hepatitis	15	+	190	70	20	4.6	640			Post mortem	Died
2	Stricture Common Bile Duct. Biliary Cirrhosis	35	+	260		14	1.7	trace	43	45	Operation	Chronic
6	Toxic Hepatitis. Pernicious Anemia	39	+	145	25	35	4.6	180	77	100		Recovered
7	Toxic Hepatitis (Alcohol)	39	+	490	145	41	1.8	40	22	100		Developed nodular cirrhosis Improved
8	Subacute Yellow Atrophy	87	—	170	30	70	2.4	4	18	100		
9	Alcoholic Cirrhosis. Hepatitis Avitaminosis	14	+	240	45	25		640	30	60	Post mortem	Died
12	Blastomycotic Cholangitis. Biliary Cirrhosis	38	+	330	105	33		2500	51	80	Operation	Became chronic
15	Carcinoma Head Pancreas. Suppurative Cholangitis	4	+								Operation	Died
16	Pyelonephritis. Bacillus Coli Bacteremia, Toxic Hepatitis	28	+	190	85	85	8.3	320	15	100		Recovered
17	Hepatitis. Nodular Cirrhosis, Alcoholism	46	+	600	290	50	2.8			100		Recovered
18	Hepatitis. Diabetes	36	+	460	110	42	10.0	10	29	100		Recovered
19	Hepatitis	38	+	240	80	40	4.0	10				Recovered
27	Hepatitis. Aseptic Peritonitis	70	—	190	65	30	13.0	4				Recovered
28	Syphilis. Cirrhosis G. I. Hemorrhage not jaundiced	100	—				10.0	10				Recovered

tests, blood bilirubin, blood cholesterol, and cholesterol ester, urine urobilinogen, and blood phosphatase. In the cases other than liver disease (Table III) there were few clinical liver tests carried out.

TABLE II

Cases Believed to Have Mild Parenchymatous Damage of the Liver

Patient No.	Clinical Diagnosis	Per Cent Detoxication	Correlation	Blood Cholesterol mg. 100 cc.	Ester mg. 100 cc.	Icterus Index	Galactose Test g. in 5 hrs.	Urine Urobilinogen Dilution	Blood Phosphatase King-Armstrong Units	Prothrombin Per Cent of Normal	Confirmation of Diagnosis	Remarks
3	Hepatitis (Alcohol)	78	—	405	196	14	5.3	640				Improved
4	Convalescent from Mild Simple Jaundice	100	—	280	155	26	0	1290				Recovered
10	Simple (Catarrhal) Jaundice	3	+	280	150	25	3.3	1240		100		Recovered
11	Splenomegalic Cirrhosis G. I. Hemorrhage (No jaundice)	39	+	145	60					100	Operation	Recovered
20	Stricture Common Duct Biliary Cirrhosis. G. I. Hemorrhage	79	—	230	75	12	0.6	4		100	Operation	Improved
23	Laennec's Cirrhosis (No jaundice)	19	+				0	10	Normal			Improved

TABLE III

Control Cases. Diseases with No or Slight Liver Parenchymatous Damage

Patient No.	Clinical Diagnosis	Per Cent Detoxication	Correlation	Blood Cholesterol mg. 100 cc.	Ester mg. 100 cc.	Icterus Index	Galactose Test g. in 5 hrs.	Urine Urobilinogen Dilution	Blood Phosphatase King-Armstrong Units	Prothrombin Per Cent of Normal	Confirmation of Diagnosis	Remarks
5	Common Duct Stone	53	—	310		36	0	5		65	Operation	Recovered
13	Rheumatic Fever	42	—									Improved
14	Rheumatic Fever	27	—									Improved
21	Common Duct Stone	45	—	320	50	15	0	4		100		Improved
22	Purpura Hemorrhagica	43	—							100		Recovered
24	Lymphogranuloma Inguinale. Ulcerative Colitis	45	—								Biopey	Improved
25	Gastric Ulcer	68	+								X-Ray	Improved
26	Common Duct Stone. Duodenal Ulcer	13	—	370	210	27					X-Ray	Improved

CONCLUSIONS

Of the 14 cases that were definitely diagnosed as severe liver damage, all but 3 showed low excretion of glucuronides, and this agreed with the clinical diagnosis. Of the 6 cases diagnosed as mild liver impairment 3 showed low glucuronide excretion. But in the other diseases in which no liver damage was believed to be present (Table III) there was almost complete lack of correlation between clinical and chemical estimates, 7 of the 8 showing abnormally low glucuronides. It is of interest to quote the following statement by Fishman (2) who worked with mice and dogs.

"The demonstration by Oshima (3) that other organs besides the liver contain high concentrations of β -glucuronidase, and confirmation of this fact in the present work, suggest that the process of glucuronic acid conjugation is by no means confined to this organ."

The ingestion of the borneol in our 26 normal and 28 pathological subjects was not attended by any unusual effects, and the borneol appeared to be well tolerated. About the only inconvenience encountered by all subjects was the odor of borneol on the breath for several hours following ingestion.

We wish to acknowledge the assistance of Mr. Morris Roberts.

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Renin-Substrate from Beef Serum

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Evidence has accumulated in the last few years that renin, a protein obtainable from kidneys, is perhaps a causative factor in the development of arterial hypertension of the essential and malignant type in human beings.

Renin itself has no direct effect on blood pressure (1) but in the circulating blood it reacts with a substance contained in plasma (renin-activator) and forms a pressor compound, named angiotonin (2) or hypertensin (3). In the system:



renin is an enzyme (1, 4, 5), renin-activator a substrate, which will be called renin-substrate in this report. The reaction product angiotonin or hypertensin is apparently a polypeptide and has physiological properties somewhat similar to those of amines of the substituted phenylethylamine type. Renin and renin-substrate are both proteins and can be separated from angiotonin in various ways, for example by the addition of 3 volumes of boiling alcohol to their solution, which will denature and precipitate the proteins while angiotonin stays in solution (6).

In a number of organs and in erythrocytes, there occur one (7) or several (8) enzymes, hypertensinase, which destroy angiotonin or hypertensin. A relatively small amount of hypertensinase has been found in plasma and serum. This enzyme is sensitive to acids and can be inactivated by keeping its solution for 30 min. at pH 3.7 at room temperature (7, 9, 10).

In an earlier paper, a method for the preparation of renin has been described (11). This purified material, which is stable in form of a dry powder, has now been incubated with beef serum and various serum

protein fractions. The amounts of angiotonin formed with each fraction were compared and the results form the basis for the preparation of a purified renin-substrate from beef serum.

EXPERIMENTAL

1. Fractional Precipitation of Serum Proteins

To 1200 ml. of ice-cold beef serum containing 94 g. (7.83 per cent) protein, were added 350 ml. water and 50 ml. 0.4 NH_4SO_4 . Saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added dropwise under constant mechanical stirring at 0°C . After the addition of about 800 ml. ammonium sulfate solution, its concentration in the mixture was determined electrophotometrically after nesslerization and found to be 1.25 molar, pH 6.4 (glass electrode). The precipitate was allowed to settle overnight in the cold and was then removed by filtering with suction through a Büchner

TABLE I
Fractionation of Beef Serum with Ammonium Sulfate
(Volume 1200 ml., Protein-Contents 7.83 per cent)

Fraction	$(\text{NH}_4)_2\text{SO}_4$ Concentration					
	Saturation	Molarity	pH	g. Prot. pptd.	g. in Dist. Water soluble	insoluble
A.	0.30	1.25	6.4	21.1	13.6	7.5
B.	0.41	1.67	6.8	20.4	15.7	4.7
C.	0.52	2.15	6.7	4.7	3.8	0.9
D.	0.67	2.76	6.8	27.6	27.6	0

funnel. The moist precipitate (Fraction A) was dissolved in 250 ml. water and dialyzed against distilled water at 3°C . until salt free. Water soluble and insoluble parts of Fraction A were separated by filtration with suction, the water insoluble residue (euglobulins) was dissolved in 0.9 per cent NaCl solution and the protein contents of both parts of Fraction A was determined by a micro Kjeldahl method.

The filtrate from Fraction A had a volume of 2225 ml. Its ammonium sulfate concentration was slowly increased in the cold and, after addition of 370 ml. saturated $(\text{NH}_4)_2\text{SO}_4$ solution found to be 1.67 molar. The resulting precipitate, Fraction B, was treated in a similar way as outlined for Fraction A. After separation of Fraction B, two more fractions, C and D, were precipitated. Table I summarizes the results.

2. Incubation with Renin

A renin solution containing 10 rabbit units (12) per ml. was prepared from a stable dry powder (11). Solutions of the various protein fractions in 0.9 per cent NaCl were prepared with a protein content of 2.5 per cent, while the original serum was used undiluted.

In the first series of incubations, 1 ml. of renin solution was added per gram protein at 37°C . and the mixture (pH 7.4-7.5) was kept at this temperature for exactly 12 min. The reaction was then stopped by the addition of 3 volumes of

boiling alcohol. After cooling the solution was filtered and concentrated in vacuo at 40°C. almost to dryness. The concentrate was diluted with water, so that 1 ml. of the resulting solution corresponded to 20 ml. original serum, calculated on the basis of the volume of serum which yielded the protein contained in the test sample.

For a second series of incubations, all protein solutions were acidified to pH 3.6-3.7 (glass electrode) and kept there at 21°C. for 35 min.; NaOH was then added until pH 7.4 was reached and after warming the solutions to 37°C., 5 rabbit units of renin were added per gram protein. The incubation was terminated after 120 min. by the addition of alcohol and the solutions were worked up as described for the 12 min. experiments. The purpose of the pH adjustment to 3.6 was to inactivate hypertensinase which if present, would destroy within 2 hours part of the angiotonin formed by the action of renin.

TABLE II

Production of Angiotonin by Incubation of Renin with Beef Serum and Some of its Water Soluble Fractions

Substrate	Units Obtained per 100 ml. of Serum	
	Incubation	
	12 min.	120 min.
Whole Serum	260	430
Fraction A	170	120
Fraction B	450	520
Fraction C	40	70
Fraction D	30	40

3. Determination of Angiotonin

The angiotonin content of the solutions after incubation was determined by their effect on the blood pressure of cats anesthetized with nembutal. The pressor response was compared with that of a standard angiotonin solution, 1 ml. of which produced a rise in blood pressure of 25-35 mm. and was chosen as unit. This standard solution was injected alternating with the sample to be tested, the amounts of which were adjusted so that its pressor effect was close to that produced by one unit.

The water insoluble portions of Fractions A, B, and C, which precipitated during dialysis, did not yield any angiotonin after the incubation with renin. Renin-substrate is therefore not an euglobulin. The relative yields on angiotonin obtained with whole serum and its water soluble fractions were as shown in Table II.

DISCUSSION

Interest in renin-substrate increased recently, after a report by Sapirstein, Southard and Ogden (13) that the injection of a serum glo-

bulin fraction into dogs in hemorrhagic shock was of beneficial effect in restoring blood pressure. This finding is a logical conclusion from earlier work of the same authors (14) who showed that in shock due to blood losses, the kidney liberates renin into the circulating blood. Administration of renin-substrate should then compensate for the exhaustion of the normally present amount and increase angiotonin formation. This in turn would cause a rise in blood pressure. The globulin fraction used by Sapirstein, *et al.* was the precipitate formed in serum by 0.4 saturation with ammonium sulfate. Recently Plentl, Page and Davis (15) reported however, that renin substrate from hog serum is soluble in 0.45 saturated (1.85 molar) ammonium sulfate and precipitated by 0.51 saturation (2.10 molar). These authors express therefore the opinion that the fraction used by Sapirstein, *et al.* would not be most efficient in the treatment of shock.

The experiments reported here show, that renin-substrate from beef serum is precipitated at pH 6.8 between 0.30 and 0.41 saturation (molarity 1.25–1.67) with ammonium sulfate.

The finding (Table II) that in the 12 min. experiments less angiotonin is produced from serum than from the corresponding amount of Fraction B, can partly be explained by the presence of hypertensinase in whole serum, which came to a large extent from red cells, as a slight hemolysis could not be avoided. After inactivation of hypertensinase, the angiotonin yield from serum increases 65 per cent above the 12 min. value, but still amounts to only 57 per cent of the yield obtained from the four water soluble fractions (430 units as compared to 750 units). It is quite possible that renin combines or reacts with other proteins besides renin-substrate, which would not yield angiotonin. As the concentration of renin-substrate in whole serum is relatively low while other proteins are forming the major portion, this effect of competitive inhibition should be noticeable. The yield on angiotonin units per gram of protein is:

	Incubation	
	12 min.	120 min.
Serum....	33	55
Water soluble fraction B	340	400

While the protein in the water soluble portion of Fraction B represents $\frac{1}{5}$ (16.7 per cent) of the total serum protein, it is 7–10 times as potent as renin-substrate as is whole serum.

The question could be asked whether the use of such a purified renin-

substrate of human origin would be of practical value for the treatment of shock. This seems unlikely. The substrate is a relatively unstable globulin and its administration would only lead to the production of angiotonin, which can be obtained in stable form and unlimited quantities *in vitro*. It would therefore be simpler to use angiotonin itself for shock treatment, or even synthetic drugs of similar action, such as paredrinol, provided that such a peripheral action is desirable in certain types of shock.

SUMMARY

1. Seven protein fractions from beef serum were prepared and tested for their ability to form angiotonin during incubation with renin.

2. The water insoluble fractions did not yield any angiotonin. Renin-substrate is therefore not an euglobulin.

3. Renin-substrate was mainly present in the fraction precipitated between 0.30 and 0.41 saturation (1.25–1.67 molarity) with ammonium sulfate at pH 6.8.

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Purification and Properties of Penatin

The Second Antibacterial Substance Produced by *Penicillium notatum* Westling*

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INTRODUCTION

The observation that the crude culture filtrate of *Penicillium notatum*, when grown on Czapek-Dox medium, has different antibacterial characteristics than does purified penicillin, led to the conclusion that another antibacterial substance must be produced by the mold (1). This second antibacterial substance which I have called penatin (2) has been purified about 1000 times and differs from penicillin in many respects (3). While penicillin is a nitrogen-containing acid (4, 5, 6, 7), soluble in many organic solvents and of low molecular weight, penatin is a compound of high molecular weight, a protein whose characteristics have not yet been sufficiently determined; it is not dialyzable through cellophane tubing, and not extractable from the crude culture by organic solvents as is penicillin. In its bacteriostatic and bactericidal properties, penatin has a far wider range than penicillin, especially against some gram negative organisms. Probably the most interesting fact about penatin is that it requires dextrose in order to be effective, something unique in the realm of antibiotic substances thus far. The most highly purified penatin preparations are yellowish powders, soluble in water but not in the common organic solvents. In the dry state penatin is stable for months, and is fairly stable in solution (pH 2-7).

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EXPERIMENTAL

Strain Employed

In the studies now described, the strain PEN 2 has been used exclusively. This strain was given to the writer by Dr. S. A. Waksman, and was marked as follows: "*Penicillium notatum* (Westling) '77'." It came from C. B. S. Baarn, Holland; it was isolated in 1925, and received in 1941. In Dr. Waksman's collection this strain is numbered "PEN 2".¹

Medium used. Optimum production of penatin has been achieved by using the modified Czapek-Dox medium (8).

Sterilization, Inoculation, Growth. 300 cc. of the modified Czapek-Dox medium are placed into Roux-bottles (Pyrex No. 570) and sterilized for 20 minutes at 121°C. The depth of the medium is about 17 mm. After cooling the bottles are inoculated rather heavily with an approximately 7 day old spore culture suspension in NaCl, obtained from malt extract agar. The incubation was 7 days at 28°C. The growth during this time—and all phenomena related to the growth of the mold, are described with the reservation of the known irregularities of the genus of *Penicillium*—is about as follows: A very thin pellicle is usually formed during the 2nd or 3rd day of incubation. On the 3rd or 4th day the pH will have reached about 3.0 and the pellicle will have become somewhat thicker, but remains white with no sporulation until the harvest day. On this (7th) day the pellicle may or may not be slightly wrinkled and slimy on the undersurface. The optimum production of penatin under those conditions is reached on the 7th day, when the mold culture is harvested. The pH is invariably at 3.5–4.0 and the color of the fluid just slightly yellowish. This yellow color deepens during the exposure incident to filtration. Occasionally a bottle will show more or less abundant spore formation of the pellicle on the 7th day or earlier. This is always associated with a brittle, greenish pellicle, strong yellow-orange pigment formation, a higher pH (6–8) and almost no penatin production. Those bottles were discarded. The possibility that a contamination might be the cause of the irregularity has not been investigated. While it has been found best to use a large number of small bottles for the culturing of the mold, it doubtless would be possible to use large trays with glass covers, since the pH of the culture liquid in no stage will be above pH 4.5. The procedure of replacing the culture fluid under the original mycelium as used by other workers (4) has never been tried.

¹ Although it seemed unimportant to describe the strains of *Penicillium notatum* used in the previous communication (1), the new findings make this necessary. The strains used in the previous work were the following: PEN 1 = *Penicillium notatum*, (Fleming strain), Thom No. 144-51121; PEN 2 = see above; PEN 3 = *Penicillium notatum* Westling "78", N.C.T.C. No. 4222; PEN 4 = see communication in (1); PEN 6 = *Penicillium notatum*, Reid strain. Those were the denominations of the strains in Dr. S. A. Waksman's collection, to whom the writer is grateful for the transfers.

From the day the cultures are harvested, all steps in the purification of penatin are carried out at ice-box temperature. The culture is filtered and stored in the ice-chest. The antibacterial potency of such a culture filtrate is remarkably stable for many days, even weeks. When the culture fluid was tested for its antibacterial properties on the day of harvesting and again 7 or 14 days later, it was always found that the antibacterial value of the solution had increased slightly, accompanied

TABLE I

Increase of the Antibacterial Potency of a Crude Culture Filtrate of Penicillium Notatum Westling

cc./plate	Days at Ice-Chest Temperature—Storage											
	0			7			14			21		
	C	B	S	C	B	S	C	B	S	C	B	S
10	—	—	—	—	—	—	—	—	—	—	—	—
0.5	—	—	—	—	—	—	—	—	—	—	—	—
0.3	—	—	—	—	—	—	—	—	—	—	—	—
0.1	1	—	—	—	—	—	—	—	—	—	—	—
0.05	3	—	—	1	—	—	±	—	—	—	—	—
0.03	3	1	±	3	—	—	3	—	—	3	—	—
0.01	3	3	3	3	3	2	3	2	±	3	±	—
0.005	3	3	3	3	3	3	3	3	3	3	3	3
pH	3.5			3.3			3.3			3.2		

Above specified amounts of crude culture fluid were incorporated into 10 cc. of tryptose agar and the test organisms streaked out on the solidified agar. Readings, after 48 hours at 37°C.—C = *E. coli*; B = *Brucella abortus*; S = *Staph. aureus*; — = no growth; ± = just visible growth; 1 = faint growth; 2 = partially inhibited growth; 3 = good growth, similar to control.

by a slight shift of the pH toward the acid side (Table I). Whether this increase of the antibacterial property was due to the acid reaction and liberation of more penatin, or to other circumstances, has not been investigated.

Method of Assay

As before (1) the streak test was used after incorporation of the antibacterial substance in 10 cc. of tryptose agar (Difco) unless stated otherwise. Readings were taken after 48 hours at 37°C.

Other Media

When to the modified Czapek-Dox medium is added corn steep liquor or yeast extract, or for the dextrose is substituted brown sugar or malt extract, the growth of the mold is accelerated, the pigment production is increased, the formation of the pellicle is different and above all, an antibacterial substance, different from penatin, is produced. The following changes were observed (Table II):

TABLE II

Influence of the Composition of the Medium upon the Formation of the Second Antibacterial Substance by Penicillium Notatum Westling

	Yeast			Malt			Brown Sugar			Modified Czapek-Dox Medium		
Days of growth at 28°C. for optimum production of antibacterial substance	6			6			6-7			7		
pH	7.4			7.1			6.6			3.5		
cc. Crude culture fluid per 10 cc. tryptose agar.	C	B	S	C	B	S	C	B	S	C	B	S
1.0	+	-	-	+	-	-	+	-	-	-	-	-
0.3	+	-	-	+	-	-	+	-	-	-	-	-
0.1	+	+	-	+	+	-	+	-	-	-	-	-
0.05	+	+	+	+	+	+	+	-	-	-	-	-
0.03	+	+	+	+	+	+	+	-	-	+	-	-
0.01	+	+	+	+	+	+	+	+	?	+	-	-
0.005	+	+	+	+	+	+	+	+	+	+	+	+

Yeast: To one liter of the modified Czapek-Dox medium was added 10 g. of yeast extract (Difco).

Malt: The dextrose in the modified Czapek-Dox medium was replaced by 40 g. of malt extract (Difco).

Brown Sugar: The medium used by Hobby, *et al.* (9) was used.

C = *Escherichia coli*; B = *Brucella abortus*; S = *Staph. aureus*.

Conduct of the antibacterial test, as mentioned in the experimental part.

+ = Full growth; - = no growth; ? = trace of growth.

Brown Sugar Medium

The medium used by Hobby, *et al.* (9) was used. The pellicle of the mold became thick and yellow and many droplets formed on the pellicle. A strong yellow pigment was formed and the pH on the 6th day was 6.6, which is the optimum for the production of an antibacterial substance different from penatin.

Yeast Extract Medium

When 10 g. of yeast extract (Difco) were added to 1 liter of the modified Czapek-Dox medium, the mold grew much faster than on Czapek-Dox medium alone; the pellicle was wrinkled; the culture fluid was dark yellow in color. The pH on the 6th day was 7.4.

Malt Extract Medium

The dextrose in the Czapek-Dox medium was replaced by 40 g. of malt extract (Difco). On the 6th day the maximum production of antibacterial substance was reached. The pellicle produced abundant dark green spores; the culture fluid was dark yellow; the pH was 7.1.

When penatin is defined with emphasis on its antibacterial properties against *Escherichia coli* especially, it is clear that all the media, except the plain modified Czapek-Dox medium, will prevent the formation of penatin. Whether or not the antibacterial substance produced on the media mentioned above is similar to penatin, has not been investigated.

Purification of Penatin

Penatin is readily adsorbed by kaolin. At first a kaolin of an unknown foreign make was used which permitted rather high recoveries of penatin. After this supply was exhausted and could not be replaced, another brand had to be substituted. The kaolin should have the following properties: It should adsorb penatin quantitatively, settle quickly and allow a quantitative elution. Apart from the last property—among many kaolins tried—Baker's kaolin ("washed and ignited"), was found to be best suited. The kaolin is prepared by washing it electrolyte-free with distilled water and diluting to 200 mg./cc. As mentioned before, all the operations from the harvest of the mold culture are carried out at refrigerator temperature. To the crude culture filtrate (which has a pH of 3.5–4.0) are added 8 g. of kaolin with slow stirring. After standing over night the supernatant liquid, which contains no penatin (1.0 cc. will give no inhibition with either *E. coli* or *Staphylococcus aureus* in

10 cc. of test medium) is siphoned off the kaolin as far as possible and the kaolin is washed with an amount of cold distilled water about equal to that of the original volume of the crude culture fluid.² Any unnecessary stirring either during the adsorption or washing has to be avoided. After the kaolin has settled over night, the wash water is siphoned off and the kaolin is eluted in about $\frac{1}{20}$ of the original volume. As eluents can be used: $M/5$ KH_2PO_4 - Na_2HPO_4 -buffer, pyridine, or (and this was used mostly) $M/5$ Na_2HPO_4 . The eluent is added to the heavy kaolin suspension, until a pH of about 6.3 is reached. For the first 2-4 hours a slight drop in the pH to the acid side is compensated by the addition of more of the eluent until the pH of 6.3 is restored. After standing over night, the kaolin is centrifuged off and to the clear yellowish solution, having a pH of 6.0-6.3, containing the penatin, is added dioxane until a 60-70 per cent concentration of dioxane is reached. Depending upon the amount of penatin eluted, a yellow precipitate will form, either immediately or after 1-2 hours standing in the ice box. After standing over night the precipitate is centrifuged off. The clear supernatant liquid, which is yellowish from traces of pigment and which contains practically no penatin activity, is discarded. The bright yellow sediment is dissolved in a small amount of water and the small amount of a white precipitate formed is centrifuged off and discarded. The supernatant yellow solution is the purified penatin. After dialysis against distilled water it can be used directly or dried in vacuo from the frozen state, and kept in the ice chest.

Losses, Stability, Purity

The kaolin removes all of the penatin and only about 10 per cent of the associated solids. The main loss of penatin occurs in the elution. Although much time was spent to increase the yield by the elution, no better method than the one reported above has been found. The yield by the elution is subject to great variation: From 10 to 100 per cent of the penatin is recovered, but the average is rarely above 50 per cent. No further detectable losses are encountered during the further operations, provided all manipulations are carried out at refrigerator temperature.

² In several attempts to elute penatin from the kaolin with phosphate and dextrose, the pH became acid over night (pH 4.5 and less) and practically no penatin was eluted. The decomposition of dextrose was explained as the action of an enzyme, present as an impurity. The discovery of the British workers (15), however, has shown otherwise.

The maximum purification achieved is about 1000 times or more, as compared on the basis of dry weight of the crude culture, which at the time of the harvest will have about 24 or 25 mg. per cc. In the dried state penatin is a light yellowish powder, stable for months in the refrigerator, somewhat hygroscopic and readily soluble in water. Penatin in the purified state, in concentrated solutions is bright yellow. All attempts aimed at the separation of the yellow color from the antibacterial substance have been unsuccessful, resulting always in the destruction of the antibacterial activity, thus leading to the conclusion that the color is inherent in penatin itself. No attempt to purify penatin further has been made, since most of the material produced had to be converted for animal experiments. Highly purified penatin is insoluble in all the common organic solvents, by which it is precipitated in 50-70 per cent solution. Penatin is inactive in the presence of small amounts of acetone and is inactivated by methyl alcohol but not by ethyl alcohol. Penatin is quite resistant to acids, withstanding for instance a pH of 2 for 24 hours at ice box temperature. At pH higher than 7 inactivation proceeds rapidly. Penatin gives positive protein reactions (biuret, Millon) and also a positive Molisch test and will not dialyze through cellophane tubing.

Other procedures to purify penatin have been tried but none better than the kaolin adsorption has been found. Penatin can be precipitated from the crude culture (pH 3.5) by tannic, phosphomolybdic and phosphotungstic acids; not by Ba and Cu salts, picric and oxalic acids. Charcoal, tricalciumphosphate, aluminum gel C₇ will not adsorb any appreciable amounts of penatin; however Fuller's earth will. The first attempt to purify penatin was made with phosphotungstic acid. Penatin can be precipitated from the crude culture with fair recovery with phosphotungstic acid, which possibly forms an acid-insoluble penatin-phosphotungstate, whereby a purification of about 20 times is achieved. This precipitate was found to be stable for over three months. The optimum concentration is from 2-5 cc. of 10 per cent phosphotungstic acid per liter. Penatin-phosphotungstate is insoluble above pH 5.6; however, the decomposition of this compound into free penatin with Ba-salts, *e. g.*, was unsatisfactory and had to be abandoned because of the losses encountered.

Mode of Antibacterial Action

The action of purified penatin on test bacteria is bacteriostatic and bactericidal, although the bacteriostatic properties seem to predominate.

The mode of action does not seem to be lytic. Table III shows the bacteriostatic and bactericidal action of penatin on living cells of *Brucella abortus*.

Table III seems to indicate that penatin is rather bacteriostatic than bactericidal, furthermore it shows, that very small quantities of the antibacterial substance might act—at least in the first stage of action—as growth stimulants rather than inhibitors as compared with the control.³

TABLE III

Bacteriostatic and Bactericidal Effect of Penatin on the Growth of Brucella Abortus

Time = hrs.	Penatin/cc.						Control
	0.4	0.2	0.1	0.05	0.03	0.01	
0							1,400
6	350	680	1,000	12,000	16,000	19,000	7,000
24		20	650	9,000	22,000	26,000	20,000
48			70	400	18,000	31,000	29,000
Streak test after 48 hrs.	—	—	—	—	±	3	

Bactericidal test: Designated amounts of penatin were added to 10 cc. of a tryptose broth culture containing a 24 hours' growth of *Brucella abortus* of an intensity as indicated in the control. After intervals of 6, 24 and 48 hours one cc. of broth was plated out in proper dilution in triplicate and the colonies developed after 72 hours of growth at 37°C. were counted. Numbers indicate average readings of surviving *Brucella abortus* cells in millions in 1 cc. of broth.

Bacteriostatic test: Streak test, same as described in Table I.

Influence of Serum

When sterile horse serum was added to a penatin preparation, purified about 200 times, in percentages of 10, 50 and 90 per cent and the mixture was allowed to stand at room temperature for 24 hours, samples taken at 4, 8, and 24 hours revealed no diminution of the antibacterial potency of penatin as compared with the control, the test organisms being *Staph. aureus*, *E. coli* and *Brucella abortus* (Streak test).

³ The last mentioned phenomenon has been observed with many other antibacterial substances, such as gliotoxin, actinomycin, streptothricin and pyocyanase. (Unpublished experiments by the author.)

Influence of Dextrose

From the experiments with dextrose, sterilized under different conditions (1) which showed the appearance and disappearance of a factor against *E. coli*, as well as from the fact that purified penatin showed activity only on media containing dextrose, it was clear that this substance must play a major rôle. When the crude culture of penatin was

TABLE IV
The Influence of Dextrose upon the Antibacterial Activity of Penatin

γ -Penatin per 10 cc.	Media													
	TA + BH			NA ¹		NA ²		NA ³		NA ⁴		TA ⁵		
	C	B	S	C	S	C	S	C	S	C	S	C	B	S
1	—	—	—	+	+	+	+	—	—	—	—	+	+	+
0.5	—	—	—	+	+	+	+	—	—	—	—	+	+	+
0.3	+	—	—	+	+	+	+	+	—	+	—	+	+	+
0.1	+	—	—	+	+	+	+	+	—	+	—	+	+	+
0.05	+	+	—	+	+	+	+	+	—	+	—	+	+	+
0.03	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Conduction of the test: as before, streak test.

TA = Tryptose agar, BH = Brain heart infusion agar (the results obtained on those two media were about the same).

NA¹ = Nutrient agar, containing no dextrose.

NA² = Nutrient agar, containing no dextrose, hundred times the amount of penatin shown in column one was added.

NA³ = Nutrient agar, containing 10 mg. dextrose per 10 cc.

NA⁴ = Nutrient agar, containing 1 mg. dextrose per 10 cc.

TA⁵ = Tryptose agar, containing no dextrose.

C = *E. coli*, B = *Brucella abortus*, S = *Staph. aureus*.

The substances mentioned as ineffective in producing an antibacterial action with penatin, are not recorded in this table, but were used in a concentration of 10 mg. per 10 cc. of medium (tryptose agar).

dialyzed long enough it was observed that penatin stayed inside the cellophane membrane and had lost all antibacterial properties. Its activity, however, could be restored by adding small amounts of the dialyzed liquid or dextrose. The following substances tested failed to impart antibacterial power to penatin: sucrose, arabinose, levulose, maltose, glycerin, alcohol, pyruvic acid, vitamin B₁, B₂, B₆, vitamin C, pantothenic and nicotinic acid. Whether dextrose is the only carbo-

hydrate able to impart to penatin its antibacterial power, is not known at the present (Table IV).

Toxicity

Various penatin preparations, ranging from 200 to 1000 times purified material, were injected intramuscularly into guinea pigs in amounts of 10 to 500 mg. Daily doses of 10 and 20 mg. per guinea pig for five days were tolerated by the test animals without apparent ill effects. Repeated doses of 50 mg. produced a temporary elevation of temperature and a swelling at the site of the injection. A single injection of 250 and 500 mg. produced temporary fever and a severe local reaction. However, none of the injected animals died as a result of the treatment. A single injection of 16.5 mg. intravenously into a rabbit did not produce any ill effects. The therapeutic effect of penatin on guinea pigs infected with *Brucella abortus* will be reported elsewhere.

DISCUSSION

It seems strange that the second antibacterial substance produced by *Penicillium notatum* should have escaped the attention of the many workers engaged in penicillin research. For *Penicillium notatum* Westling '77' is not the only strain found capable of producing a second antibacterial substance, as has been indicated before (1), and as is also evident from other unpublished experiments. Of five strains of *Penicillium notatum* tested, all were found to be producing a second antibacterial substance, different from penicillin.

While this paper was in preparation a publication by Raistrick *et al.* appeared (15) entitled, "Notatin: an antibacterial glucose-aerodehydrogenase from *Penicillium notatum* Westling," characterizing the second antibacterial substance produced by *Penicillium notatum* Westling as a yellow enzyme.

In another recent paper E. A. Doisy and collaborators (16) describe another antibacterial substance derived from *Penicillium notatum*, "Penicillin B." Although this substance has many similarities with penatin or notatin, it appears to differ in certain respects: Penicillin B derives from a different strain of *Penicillium notatum* than does penatin. Penicillin B shows toxic effects in animal experiments; it has a lower stability in crude culture and is formed in media unsuitable for the production of penatin.

The discovery of the British workers (15) that notatin is a yellow

enzyme brings up the question—apart from many other considerations—whether penatin and notatin are identical. No definite proof of this can be given at this time, although from many facts (in both instances a

TABLE V

Bacteriostatic Action of Penatin against Various Pathogenic and Non-pathogenic Organisms

Test organisms	Dilution 1:(millions)					
	12.5	25	42	125	250	420 1250
<i>Sarcina lutea</i>	—	—	—	—	—	Tr N
<i>Gaffkya tetragena</i>	—	—	—	—	—	I N
<i>N. catarrhalis</i>	—	—	—	—	—	I N
<i>Staph. aureus</i>	—	—	—	—	Tr N	N N
<i>Staph. albus</i>	—	—	—	—	N N	N N
<i>Staph. aureus</i>	—	—	—	Tr N	N N	N N
<i>C. diphtheriae</i>	—	—	—	I N	N N	N N
<i>Cl. histolyticum</i>	—	—	—	I N	N N	N N
<i>B. brevis</i>	—	—	—	I N	N N	N N
<i>Eb. typhosa</i>	—	—	—	N N	N N	N N
<i>B. anthracis</i>	—	—	Tr N	N N	N N	N N
<i>Br. melitensis</i>	—	—	Tr N	N N	N N	N N
<i>B. mycoides</i>	—	—	Tr N	N N	N N	N N
<i>Act. lavendulac</i>	—	—	—	N N	N N	N N
<i>Ps. aeruginosa</i>	—	—	—	N N	N N	N N
<i>Br. abortus</i>	—	—	Tr N	N N	N N	N N
<i>B. subtilis</i>	—	—	Tr N	N N	N N	N N
<i>S. paratyphi</i>	—	—	I N	N N	N N	N N
<i>D. pneumoniae</i> Type I.....	—	Tr I	N N	N N	N N	N N
<i>S. pyogenes</i> (C 203M).....	—	Tr N	N N	N N	N N	N N
<i>Aerob. aerogenes</i>	—	—	I N	N N	N N	N N
<i>Es. coli</i>	—	I N	N N	N N	N N	N N
<i>Trichophyton interdigitale</i> *.....	—	I N	N N	N N	N N	N N

Conduct of the test: Penatin was incorporated in tryptose agar and the test organisms streaked out. Readings after 48 hours at 28 or 37°C. respectively.

— = no growth, Tr = trace of growth, I = inhibited growth, N = normal growth, similar to control.

* Krainsky's agar was used. Readings after 10 days at room temperature.

In addition to the organisms shown in this table 3 molds, 4 actinomycetes and 16 bacteria, all soil organisms, picked at random, were tested against penatin and were found to be inhibited in dilutions, ranging from 25–50 millions.

strain of *Penicillium notatum* Westling was used for the production of the substance) mentioned in this paper, it seems that penatin and notatin are probably closely related, if not identical. Thus penatin will for

instance decompose glucose with the formation of an acid, as yet unidentified, which will not reduce Fehlings solution, and hydrogen peroxide.

In the light of the discovery by the British workers many peculiarities of penatin are clarified. Furthermore the "suppression" of penatin formation in the presence of yeast or malt extract and brown sugar may find its explanation in that substances present in the above preparations may compete as active groups for the protein part of penatin, thus resulting in the formation of antibacterial substances, with different antibacterial properties.

The discovery that notatin is a yellow enzyme indicates an important link between enzymes, vitamins and "antibacterial substances." In this case the antibacterial substance has been shown to be at the same time a necessary metabolite in the organism, capable of fulfilling two different functions at least.

This dual function of enzyme and antibacterial substance brings up the question whether also other so-called antibacterial substances of microbial origin might be considered primarily as metabolites in the life of the organism, perhaps as more or less dissociated active groups (co-enzymes) of protein carriers, a view the writer has always favored and discussed but never before ventured to put in print.

Among the many as yet unexplained facts regarding the production of antibacterial substances by micro-organisms is the apparent dual nature of these substances. Among the organisms studied in detail at least three have been shown to produce two different antibacterial substances. Hotchkiss and Dubos (13) found tyrocidine and gramicidine produced by *Bacillus brevis*, and Waksman and Woodruff (14) actinomycin A and B, produced by *Actinomyces antibioticus*. The third case is the one reported in this paper. It would be interesting to know whether there is an interaction of some kind between those two substances.

The fact that thus far no organism has been found which will resist the antibacterial action of penatin is unusual among all the described antibiotic substances of soil origin. It might seem that either penatin has a configuration which will not permit enzymatic attack by the test organism, in contrast to penicillin, which is split by an enzyme (10); or that the mechanism of the antibacterial action of penatin is different—one is inclined to say "unspecific"—from the action of other antibacterial substances known. If this be true, it might seem feasible to try out

penatin not only against bacteria, but also against other disease producing agents. Since penatin will prevent the growth of gram positive and gram negative organisms, the theory of Dubos and Hotchkiss (11) may have to be reexamined. Thus another substance, derived from an actinomyces of the lavendulae type isolated by the writer (2) and described in detail by Waksman and Woodruff (12) as streptothricin, destroys living cells of *E. coli* and *Staph. aureus* with about the same intensity.⁴

One of the difficulties encountered during this work was the lack of a uniform and standardized antibacterial test, enabling one to compare results obtained with results in other institutions. Although the desire to express the antibacterial action of penatin in terms of "units" was great, it was not undertaken in order not to increase the many different "unit"-denominations already in existence. Any kind of simple standardized antibacterial test—even though it might have to be abandoned or modified in the light of future research—will certainly greatly facilitate a direct comparison of results obtained in the different laboratories. It has been thought that if for instance at least two "standardized" test-organisms, *i.e.*, *E. coli* and *Staph. aureus*, obtainable from the American Type Culture Collection, used on standardized media under conditions agreed to by the majority would be of great help to all concerned. The writer is eager to receive any comment on this proposal or any other suggestion.

SUMMARY

1. An approximately 1000 fold purification of penatin, the second antibacterial substance produced by *Penicillium notatum* Westling is described.

2. Highly purified penatin gives protein reactions, and displays its antibacterial properties only in the presence of dextrose which is decomposed by penatin with hydrogen peroxide formation.

3. Penatin is highly bacteriostatic and bactericidal against many pathogenic and non-pathogenic microorganisms, especially against gram-negative bacteria not appreciably susceptible to the action of penicillin.

4. The production of penatin is suppressed by the addition of certain substances to the Czapek-Dox medium.

5. Fairly large amounts of purified penatin can be tolerated by test

⁴ Unpublished experiments by the author.

animals when injected intramuscularly. There are indications that small amounts of penatin can be injected intravenously without obvious ill effects.

6. Penatin is in all probability very similar to if not identical with notatin.

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On the Nature of Local Anesthesia

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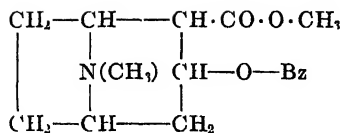
INTRODUCTION

The development during the last forty years of a wide variety of local anesthetics presents a classic example of the successful application of chemistry to medicine. From the elucidation of the structure of cocaine to the synthesis and application of such widely used substances as procaine, butesin, and orthoform represents a large amount of synthetic chemical work and innumerable biological trials. But although much of practical benefit has resulted from this work, it has not been possible to draw many conclusions of a general theoretical nature.

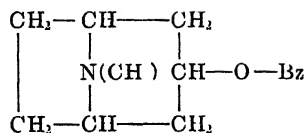
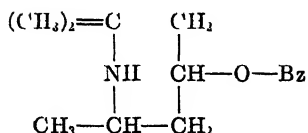
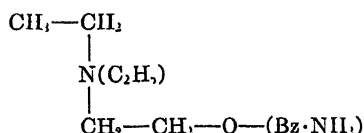
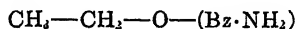
DISCUSSION

In this field there are two fundamental questions: (1) What is the essential relation between anesthetic action and chemical structure?; and (2), What is the biological mechanism of local anesthesia? The first of these questions has been studied and partially answered through the synthetic work of Einhorn and many others (see, *e.g.*, the reviews by Hirschfelder and Bieter (1932) and by Cook (1938)). It is the purpose of this paper to suggest an answer to the second question.

The study of cocaine and its analogues led, early in this century, to the view that while neither of the ring structures of cocaine is essential to anesthetic action, the benzoyl (or the *p*-amino-benzoyl) ester grouping is of critical importance. The activity of this group is greatly increased by the presence of an alkylamino group situated at 2, 3, or 4 carbon atoms distance from the benzene ring. The successive stages of simplification which led to these conclusions can be summarized by the following series of substances (Bz = Benzoyl):



I. Cocaine

II Tropacocaine
(carboxymethyl removed)III. β -Eucaine
(one ring opened)IV Procaine or Novocaine
(both rings opened)

V. Anesthesin

Of these, the first four are powerful local anesthetics and the last is weaker, but useful on account of its ability to penetrate the skin. It may be concluded, leaving aside all secondary complications, that the most effective grouping is:

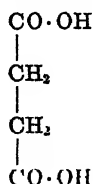


VI.

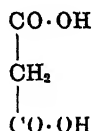
where R is an alkyl group, and $(\text{Bz}\cdot\text{NH}_2)$ is *p*-amino-benzoyl.

With this information, the problem is: How does such a structure exert local anesthetic action?

An approach to this may be made from consideration of similar problems in other fields. Perhaps the most clear-cut relationship between structure and poisoning effect is that elucidated by Quastel and Wooldridge (1928). The enzyme succinic dehydrogenase is poisoned very specifically and completely by malonic acid, and this effect is explained by the structural similarity between succinic acid, which is the normal substrate of the enzyme, and malonic acid:



Succinic acid

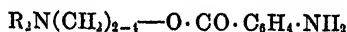


Malonic acid

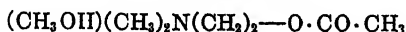
The action of the enzyme is presumably first to combine with the succinic acid, then to set free two hydrogen atoms from it, and finally to release the dehydrogenated product, fumaric acid. The similarity in molecular structure enables the enzyme to combine with the malonic acid, but since this cannot be dehydrogenated it occupies a place on the enzyme molecule to the exclusion of the succinic acid. The result is that the dehydrogenation of succinic acid is prevented. The effect is thus directly due to the structural similarity between the normal substrate of the enzyme and the poison.

The malonic acid poisoning is not an isolated case. Among other instances of the dependence of biological activity on structural similarity, the correspondence between the spatial structures of stilbestrol and the estrogenic steroids (Dodds, *et al.*, 1939), and the competition between sulfanilamide derivatives and *p*-amino-benzoic acid (Woods, 1940) may be mentioned.

From these facts it is but a short step to the suggestion that, as a general principle, specific pharmacological activity is determined by structural resemblance between the active substance and the natural substrate. To apply this principle to the local anesthetics we must ask: To what natural substrate do the local anesthetics show structural resemblance? The answer is clear; it is acetyl choline.



VI. Local anesthetic



VII. Acetyl choline

It is suggested that, in the cholinergic nerve, stimulation at the free nerve termination, or at the synapse, is produced by the combination of acetyl choline with a receptor molecule, the combination being made possible by the structural arrangement of the substrate. The nature of the "receptive substance" (Goodman and Gilman, 1940) is unknown. On this view, *the local anesthetic is a substance of sufficient structural similarity to be able to combine with the same receptor.* Although the combination does not produce stimulation, it does prevent a molecule of acetyl choline from reaching the receptor. The result is insensitivity to stimulation, or, in the more usual term, anesthesia.

The following points must be considered in connection with the proposed explanation:

1. There is at present no other satisfactory theory as to the mode of action of local anesthetics.

2. At least in the case of atropine, as pointed out by Goodman and Gilman (1940, p. 341), it is clear that the *release* of acetyl choline is not interfered with,—it is its effect which is prevented.

3. Certain experiments provide direct support for the theory. It was shown first by Liljestrand and Magnus (1919), and since confirmed by several other workers, that procaine reduces the response of muscle to stimulation of its motor nerve, while not affecting the response to direct electrical excitation. Harvey (1939) indeed showed directly that the contraction caused by injection of acetyl choline is greatly diminished by procaine, and that the same is true after denervation. In other words, procaine prevents acetyl choline from acting. Perfusion of the superior cervical ganglion with procaine completely abolishes the response both to preganglionic stimulation and to acetyl choline itself. It is worth noting that this result closely resembles the effect of curarine, as shown by Brown and Feldberg (1935), which also abolishes the response to acetyl choline. However, Harvey found that in this ganglion procaine also interferes with the liberation of acetyl choline during preganglionic stimulation. This effect on the liberation of acetyl choline complicates the situation somewhat, but it remains clear that the main action of procaine is to interfere specifically with the acetyl choline receptor mechanism.

4. It was pointed out above that, as between the alkylamino and the benzoyl-ester groups in the anesthetic, the latter is definitely the more important. Correspondingly, in acetyl choline, it is the ester which is of major importance, since hydrolysis by choline esterase inactivates it completely. Indeed, the chemical theory of nerve action makes the action of choline esterase of paramount importance. The parallelism between the functional groups of acetyl choline and of the local anesthetics is thus very close.

5. The most serious difficulty for the theory is the fact, discovered by Wedensky (1903), that a section of a nerve trunk can be "blocked" or anesthetized by cocaine. It is usually supposed that while conduction across the synapse may be due to acetyl choline, conduction along the fiber is a quite different phenomenon. However, Erlanger (1939) has brought forward a good deal of evidence to show that there is no fundamental difference between the two types of conduction. Larrabee, Gaylor, and Bronk (1939) have also shown that the two types of transmission are equally sensitive to lack of oxygen. Such experiments

open the way to the suggestion that perhaps transmission along the fiber also in some way involves acetyl choline. Indeed this view, at first reached tentatively by the author as a consequence of the present theory of local anesthetic action, finds striking confirmation from another source. Reasoning from experiments on the distribution and activity of choline esterase (Nachmansohn and Meyerhof, 1941), it is specifically suggested by Nachmansohn (1941) "that acetyl choline is intrinsically connected with the electrical changes occurring during nerve activity at the neuronal surface." While the author has neither desire nor competence to take part in the classical controversy over conduction in nerve, it seems clear that the conclusions to be drawn from the theory are supported by some of the most recent neurophysiological work.

6. The case of the sulfanilamide derivatives mentioned above provides a remarkable parallel. For here the bacteriostatic action is generally considered to rest upon competition with *p*-amino-benzoic acid, and Woods (1940) has shown that procaine, since it is a *p*-amino-benzoic acid ester, prevents the bacteriostatic action of sulfanilamide. The work has been extended by Keltch, Baker, Krah, and Clowes (1941) to seven local anesthetics derived from *p*-amino-benzoic acid, all of which counteracted the bacteriostatic effect of sulfapyridine, while other substances, also anesthetics but not related to *p*-amino-benzoic acid, had no such effect. Evidently, while the rest of the molecule remains the same, the competition here is between the sulfonamide group and the carboxylic group.

The theory here put forward deals with the problem in its simplest terms. It does not explain specific differences between the various local anesthetics. If it is supported it will provide an outstanding example of the general principle that biologically active substances will compete for a substrate if their structures are sufficiently similar, a principle which may perhaps be termed that of "structural imitation" in biological activity. One immediate result is to suggest the chemical basis along which a search might be made for substances showing structural resemblance to adrenalinic, and more effective than ergotoxine, so as to act as local anesthetics for adrenergic fibers.

SUMMARY

1. It is pointed out that the structures of the known local anesthetics may be generalized to a form which closely resembles the structure of acetyl choline.

2. The theory is therefore put forward that local anesthetics owe their ability to compete with acetyl choline for the receptive substance at nerve endings, which is the normal substrate upon which acetyl choline acts.

3. The theory is supported both by the known experimental data and by analogies drawn from other fields of biochemistry, and certain apparent objections are shown to be not inconsistent with it.

The author desires to thank Professors Henry K. Beecher, Walter B. Cannon, and G. H. Parker for critical comments and suggestions.

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A Note on the Amino Acids Yielded by β -Lactoglobulin

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A sample of crystalline β -lactoglobulin obtained from Professor R. Keith Cannan has been analyzed for nitrogen, sulfur, cystine, arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, threonine, isoleucine, valine, and leucine.

EXPERIMENTAL

The moist sample of lactoglobulin was extracted with alcohol and benzene and dried to constant weight.

Nitrogen was determined by the micro-Kjeldahl and micro-Dumas methods. Sulfur was determined by the Pregl procedure. Arginine, histidine, and lysine were isolated from four separate hydrolyzates by the modified Kossel procedure (1). Arginine was isolated as the flavianate, histidine as the nitrilate, and lysine as the picrate. The purity of each compound was established. Cystine was estimated by both the Folin (2, *cf.* 1) and Fleming-Vassel (3) methods. The Folin (4) and Lugg (5) methods were both used to estimate tyrosine and tryptophan. Phenylalanine was estimated by a slight modification of the Kapeller-Adler procedure (*cf.* 1) while threonine was determined by a combination of the *p*-hydroxydiphenyl and periodate methods (6, 7). The approximate quantities of leucine, isoleucine, and valine were estimated by Block, Bolling, and Kondritzer's (8) modification of the procedure of Fromageot and Heitz (9).

In an effort to account for unavoidable losses during hydrolysis, recovery experiments consisting of the addition of the pure amino acid both before and after hydrolysis, were carried out in the analyses of cystine, tyrosine, tryptophan, phenylalanine, threonine, isoleucine, valine, and leucine. To account for "overall" losses inherent in all isolation methods, control experiments with known mixtures of arginine,

histidine, and lysine were also conducted. No losses were observed in the hydrolysis of cystine, tryptophan, threonine, isoleucine, leucine, and valine under the conditions employed in these experiments.

The analytical values, together with results from the literature, all of which appeared after our experiments had been completed, are given in Table I.

TABLE I
Some Amino Acids Yielded by β -Lactoglobulin

Substance	Authors' per cent		Literature per cent
Nitrogen.....	15.53		15.6 (10)
Sulfur.....	1.68		1.60 (11)
	uncorrected	corrected	
Arginine.....	2.45	3.2	2.8 (12)
Histidine.....	1.34	1.8	
Lysine.....	9.24	9.9	10.6 (12)
Cystine.....	3.48 ± 0.19		3.4 (11)
Tyrosine...	3.72 ± 0.07	4.2	3.8 (11)
Tryptophan.....	1.89 ± 0.16		1.9 (11)
Phenylalanine.....	4.8 ± 0.3	5.2	
Threonine.....	5.8 ± 0.3		5.5-6.0 (13)
Isoleucine.....	6.4 ± 0.6		
Leucine.....	17.0 ± 4.0		
Valine.....	7.6 ± 1.3		

SUMMARY

β -Lactoglobulin yields 15.53 per cent of nitrogen, 1.68 per cent of sulfur, and approximately 3.5 per cent of cystine, 3.2 per cent of arginine, 1.8 per cent of histidine, 9.9 per cent of lysine, 4.2 per cent of tyrosine, 1.9 per cent of tryptophan, 5.2 per cent of phenylalanine, 5.8 per cent of threonine, 6.4 per cent of isoleucine, 7 to 8 per cent of valine, and 15 to 20 per cent of leucine.

ACKNOWLEDGEMENT

We are indebted to Miss Constance Warren, Ed.D., President of Sarah Lawrence College for permission to carry out the experiments in the College Laboratories, and to Drs. D. D. Van Slyke and R. K. Canan for their many helpful suggestions. The micro-Dumas, Pregl-sulfur, and ash analyses were carried out by Mr. J. F. Alicino of Fordham University.

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Assimilation of Acetic and Succinic Acids Containing Heavy Carbon by *Aerobacter indologenes*¹

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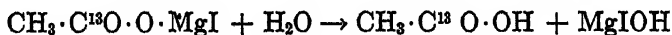
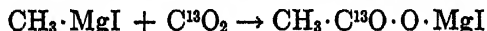
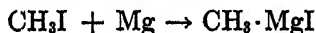
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Slade, *et al.* (1942) have shown the fixation of carbon dioxide in the carboxyl group of acetic acid during the fermentation of glucose by *Aerobacter indologenes* and *Clostridium welchii*. It was suggested that the acetic acid arose by a cleavage of a C₄ dicarboxylic acid containing fixed carbon in the carboxyl groups, into two C₂ molecules. It has been the purpose of this investigation to prove the occurrence of this reaction by the addition of synthetic acetic acid and biologically-formed succinic acid, both containing heavy carbon as a tracer, to fermentations of glucose by cell suspensions of *A. indologenes*.

The first positive proof has been obtained for (1) the formation of succinic acid by the condensation of two C₂ molecules, those of acetic acid, or a C₂ derivative, and (2) the reverse reaction, *i.e.*, the cleavage of succinic acid into two C₂ molecules which are isolated as acetic acid. In addition, positive evidence has been obtained for the (3) reduction and condensation of acetic acid to 2,3-butylene glycol and (4) reduction of acetic acid to ethyl alcohol.

EXPERIMENTAL

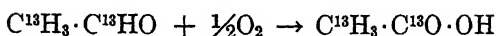
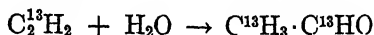
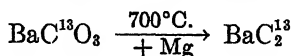
Two types of acetic acid were synthesized; the heavy isotope of carbon (C¹³) was contained in (1) the carboxyl group (type 1) and (2) in both the methyl and carboxyl groups (type 2). Type 1 acetate was synthesized according to the following reactions:



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Type 2 acetic acid was synthesized by a modification of the method of Cramer and Kistiakowsky (1941). The reactions are as follows:



Methods for the concentration and determination of C^{13} have been published elsewhere (Slade, *et al.*, 1942). All naturally occurring organic materials contain $1.09 \pm .02$ per cent C^{13} .

Aerobacter indologenes (23B) was grown for 24 hours at 30°C . on a medium consisting of 1 per cent corn sugar, 0.3 per cent peptone, 0.3 per cent $(\text{NH}_4)_2\text{SO}_4$, 1 per cent K_2HPO_4 , 10 per cent tap water, and distilled water to volume. Bacterial cells were removed from the culture by centrifugation, washed once, and resuspended in distilled water.

The experiments listed in Table I were conducted under an atmosphere of nitrogen in 0.1 *M* phosphate buffer (pH 6.2) in the presence of 2 per cent cell suspension. The experiment to which succinic acid was added contained 0.05 *M* glucose in a total volume of reaction mixture of 30 ml.; that to which type 1 acetate was added contained 0.05 *M* glucose in 60 ml.; and that to which type 2 acetate was added contained 0.1 *M* glucose in 60 ml.

Succinic acid, containing C^{13} exclusively in the carboxyl groups, was obtained as the silver salt from bacterial fermentations, acidified and extracted with ether. The acetic and succinic acids were neutralized to brom-thymol blue with a carbonate-free solution of NaOH before being added to the reaction mixtures.

Condensation of Acetic Acid to Succinic Acid and the Reverse Reaction

The addition of succinic acid containing 1.57 per cent C^{13} to fermentations of glucose by *Aerobacter* resulted in the formation of acetic acid containing 1.22 per cent C^{13} (Table I). This result proves a cleavage of the succinate molecule into acetic acid or into two molecules of a C_2 compound which are converted into acetic acid.

The formation of acetate from succinate by way of the C_4 dicarboxylic acids, *i.e.*, by oxidation of the succinate to pyruvic acid, would result in

the formation of acetate not containing C^{13} because the carboxyl groups of the succinate would be converted to $C^{13}O_2$ in the oxidation process. Also, a necessary reaction in this process, *i.e.*, oxidation of succinate to fumarate, has never been shown to occur under anaerobic conditions. Thus, if C^{13} -acetic acid cannot be formed from succinic acid by a removal of C_1 compounds, the only possibility remaining is a process involving a cleavage of the succinate to two C_2 compounds.

If acetic acid were formed entirely by a cleavage of succinate, the acetic acid should contain approximately the same per cent C^{13} as the final succinic acid. The acetic acid contained 1.22 per cent C^{13} while the

TABLE I
*Fermentation of Glucose plus Organic Acids by Cell Suspensions of
Aerobacter Indologenes*

	Acid added	CO ₂	H ₂	Formic acid	Acetic acid	Lactic acid	Succinic acid	Ethyl alcohol	2,3-Butylene glycol	Carbon recovered %	O/R Index
Control mM	0	149.0	48.5	18.7	3.4	1.2	18.1	56.0	58.6	99.5	1.00
CH ₃ ·C ¹³ O·OH mM	68.6	186.0	50.1	4.0	35.3	3.5	11.0	51.4	70.1	94.1	1.10
% C ¹³	<i>2.39</i>	1.08			<i>2.01</i>		<i>1.21</i>	<i>1.64</i>	<i>1.21</i>		
C ¹³ H ₃ ·C ¹³ O·OH mM	64.6	179.8	45.3	4.3	26.2	2.0	12.2	54.3	69.6	92.7	1.04
% C ¹³	<i>4.51</i>	1.12		1.10	<i>3.27</i>	1.07	<i>1.33</i>	<i>2.19</i>	<i>1.24</i>		
HO·OC·CH ₂ ·CH ₂ ·C ¹³ O·OH mM	130.0	133.0			5.3		126.0	6.6	44.6		
% C ¹³	<i>1.57</i>	1.09			<i>1.22</i>		<i>1.37</i>	<i>1.18</i>	<i>1.15</i>		

Products expressed as mM per 100 mM of glucose fermented.

Italicized values indicate excess abundance of C^{13} .

final succinate contained 1.37 per cent (Table I). It is probable that acetic acid was formed in this experiment by an oxidation of pyruvate as well as a cleavage of succinate. The question arises as to what extent was acetic formed by the latter mechanism. By means of calculations it will be shown that the reaction is of quantitative significance. The calculations have been made on the assumption that the C^{13} content of the final succinic acid represents the concentration of C^{13} in the succinic acid available to the cell for the cleavage reaction. It is admitted that this value involves an assumption. There is no assurance that the succinic acid formed in the cell comes completely to equilibrium with the succinate dissolved in the medium before being utilized. Undoubtedly,

cell permeability is an important factor in this respect. However, the calculation may be made in the following manner: let

X = fraction acetic acid formed other than by cleavage of succinic acid,
and

$1 - X$ = fraction acetic acid formed by cleavage of succinic acid.

The equation is,

per cent C^{13} in final acetic acid = $1.09 X + \text{per cent } C^{13} \text{ in final succinic acid} \times (1 - X)$.

Using the values expressed in Table I,

$$1.22 = 1.09 X + 1.37 (1 - X),$$

$$X = 0.53,$$

and

$$1 - X = 0.47.$$

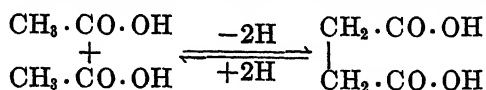
This would mean that 53 per cent of the acetic acid was formed from a substrate containing normal carbon, and 47 per cent was formed from the succinate containing C^{13} carbon. In other words, if there were 100 molecules of acetic acid, 53 of which were formed from normal carbon, and 47 of which were formed from succinate containing 1.37% C^{13} , a value of 1.22 per cent C^{13} would be obtained for the final acetic acid. Hence, under the experimental conditions, apparently one-half of the acetate was formed by a cleavage of the succinate.

A net loss of 4 mM³ of added succinate occurred during the dissimilation (cf. Table I). Reynolds, *et al.* (1937) have reported a decrease of 15 mM of added succinate per 100 mM of glucose fermented by growing cells of *Aerobacter*. In the present experiment, however, succinate was formed from the glucose. This is evident because the concentration of C^{13} in the succinate decreased from 1.57 to 1.37 per cent. Slade, *et al.* (1942) have shown the formation of succinate by *Aerobacter* by means of the Wood and Werkman reaction.

The addition of $CH_3 \cdot C^{13}O \cdot OH$ resulted in its condensation to succinic acid. The added acetate contained 2.39 per cent C^{13} and the succinate formed contained 1.21 per cent C^{13} (cf. Table I). If succinic acid was formed in this dissimilation by acetic acid condensation, the succinate should contain C^{13} exclusively in the carboxyl groups. Table II shows that the degradation of the succinic acid (cf. Slade, *et al.*, 1942) resulted in the location of the C^{13} exclusively in the carboxyl groups (1.25 per cent), while the methylene carbon atoms were negative (1.10 per cent). Hence this result demonstrates that the succinic acid was formed by

³ mM = Millimoles.

means of a carbon to carbon linkage involving the methyl carbon atom of acetic acid. This reaction may be represented as follows:



The above reaction represents C_2 condensation and is not intended to express a clear-cut picture of the reaction. From the results of these experiments it is not possible to determine the actual nature of the C_2

TABLE II

Location of the Heavy Carbon of Assimilated Acetic Acid in Compounds Synthesized by Aerobacter Indologenes

Product isolated	$\text{CH}_3 \cdot \text{C}^{13}\text{O} \cdot \text{OH}$ added (type 1)	$\text{C}^{13}\text{H}_3 \cdot \text{C}^{13}\text{O} \cdot \text{OH}$ added (type 2)
Succinic acid	1.21	1.33
Methylene carbon	1.10	1.32
Carboxyl carbon ..	1.25 (1.31)	1.29
2,3-Butylene glycol....	1.21	1.24
Methyl carbon.....	1.10	1.23
Hydroxyl carbon..	1.27 (1.33)	1.22
Ethyl alcohol ...	1.64	2.19
Methyl carbon..		2.16
Hydroxyl carbon		2.17

The values are given in per cent C^{13} . The values in parentheses were calculated by the following equation: $2X + (2 \times 1.09) = 4 \times$ per cent C^{13} in whole molecule, X = average per cent C^{13} in carboxyl carbon atoms of succinic acid or hydroxyl carbon atoms of 2,3-butylene glycol.

molecules involved in the condensation reaction. It is positive, however, that acetic acid or a C_2 derivative is involved.

The succinic acid was not formed by a synthesis of pyruvic acid by way of C'_1 and C'_2 ($\text{C}^{13}\text{H}_3 \cdot \text{C}^{13}\text{O} \cdot \text{OH}$) addition, followed by C'_3 and C'_1 addition to oxalacetic acid; for this would have resulted in the formation of succinic acid containing C^{13} in the methylene carbon atoms. Actually, the succinate contained C^{13} exclusively in the carboxyl carbon atoms (cf. Table II). Also, the cleavage of succinate to acetate supports the proposal of the formation of succinate by C'_2 and C_2 addition rather than by C_2 and C_3 addition. There is no mechanism known for the oxidation of succinate by which single carbon atoms may be split off leaving a C_2 residue

containing a carboxyl carbon atom of the original succinate. The most plausible explanation of the formation of C^{13} acetate from succinate is cleavage of succinate to two C_2 fragments by a mechanism which is the reverse of acetic acid condensation.

Calculations to show the amount of succinate formed by acetic acid condensation may be made in a manner similar to that shown previously with added succinate. Let

X = fraction succinic acid formed by a mechanism not involving acetic acid condensation, and

$1 - X$ = fraction succinic acid formed from acetic acid by condensation.

The equation is,

per cent C^{13} in final succinic acid = $1.09 X$ + per cent C^{13} in final acetic acid $\times (1 - X)$.

Using the values expressed in Table I,

$$1.21 = 1.09 X + 2.01 (1 - X).$$

This means that 13 per cent of the succinic acid was formed by C_2 condensation and 87 per cent by other mechanisms. Thus this approximate calculation shows that acetic acid condensation is a reaction which possesses quantitative significance.

On addition of type 2 acetate, succinic acid was formed containing C^{13} (Table I). The condensation of two molecules of acetic acid containing C^{13} in both methyl and carboxyl groups, would result in the formation of succinate containing C^{13} in both methyl and carboxyl groups. Table II shows that the succinate (1.33 per cent C^{13}) formed in the presence of type 2 acetate contained C^{13} equally distributed between the methylene carbon atoms (1.32 per cent C^{13}) and the carboxyl carbon atoms (1.29 per cent C^{13}). The fact that the methylene carbon atoms of succinate contained C^{13} is conclusive proof that the succinate was not formed by the CO_2 fixation reaction of Wood and Werkman, *i.e.*, the acetic acid was not oxidized to CO_2 and the resulting heavy carbon fixed in the C_4 dicarboxylic acids. If this reaction had occurred the C^{13} would be exclusively in the carboxyl carbon atoms. It is thus positively established that under anaerobiosis there exists at least two mechanisms of succinic acid formation, one involving fixation of $(C^{13})_2$, and the other condensation of acetic acid.

In this experiment in which type 2 acetate was added 11 per cent of the succinate was calculated to be formed by C_2 condensation. This value agrees with the amount of succinate formed on the addition of type 1

acetate (13 per cent). The calculation of the equilibrium constant for the condensation reaction must await the isolation of the cell-free enzyme system and the consequent elimination of the various "side" reactions which existed in the present experiments.

A net decrease in added acetate occurred in both experiments; 33.3 mM in the case of type 1 acetate, and 38.4 mM in the case of type 2 acetate. However, there has been a simultaneous production of acetic acid from the glucose. This is evident from the C^{13} content of the initial and final acetic acids. The per cent C^{13} in type 1 acetate was diluted from 2.39 to 2.01, and type 2 acetate was diluted from 4.51 to 3.27 per cent C^{13} . Mickelson and Werkman (1939) have shown a loss of 36 mM of acetic acid added to a fermentation of glucose by growing cells of *Aerobacter*.

Slade, *et al.* (1942) have shown that in the fermentation of glucose *Clostridium welchii* forms acetic acid which contains fixed $C^{13}O_2$ in the carboxyl group, although no succinate is formed. In view of the formation of acetate from succinate by *Aerobacter*, it would be important to determine whether *Cl. welchii* is able to perform the same reaction; if not, an additional mechanism of formation of acetate in that fermentation would have to be assumed.

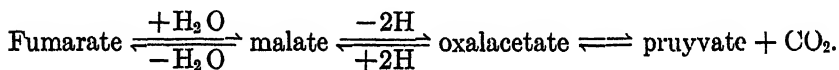
The oxidative condensation of acetic acid to succinic acid requires the participation of a hydrogen acceptor. The identity of such a molecule in these experiments is not known. However, the increased yield of 2,3-butylenic glycol on the addition of acetate, indicates that the reduction and condensation of the latter to the glycol, may serve as hydrogen acceptor for the condensation of acetic acid to succinate. Also, it has been found (unpublished data) that the addition of acetate to fermentations of oxalacetate by *Citrobacter* results in an increase in the yield of succinate. The condensation of acetate may take place under anaerobic conditions with the participation of oxalacetate as a hydrogen acceptor.

Krebs and Eggleston (1941) in experiments with *Propionibacterium shermanii* reject the possibility that succinate can be formed by a condensation of acetate, but they were able to demonstrate an increase in succinate on the addition of acetate to fermentations of oxalacetate. They believe that acetate may be oxidized to CO_2 with the reduction of oxalacetate to succinate, although no evidence exists for the anaerobic oxidation of acetate to CO_2 , and no possible mechanisms were suggested. Such an oxidation in the present experiments would have resulted in the formation of $C^{13}O_2$, and also an unequal distribution of the C^{13} between

the methylene and carboxyl carbon atoms of the succinate formed on the addition of type 2 acetate.

Wood, Stone, and Werkman (1937) have shown the utilization of acetic and succinic acids during fermentation of glucose by *Propionibacterium*. The suggestion was made that succinic acid was formed by acetic acid condensation and the former compound decarboxylated to propionic acid and CO₂. Wood and Werkman (1940) in experiments with *Propionibacterium* on glucose and glycerol, suggested that, in addition to the succinate formed by C₂ and C₁ condensation, a mechanism was also present which involved acetic or pyruvic acid condensation.

Slade, *et al.* (1942) have shown that C¹³O₂ was fixed in the carboxyl carbon atoms of lactic acid formed during the fermentation of glucose by *Aerobacter*. The possibility was presented that the lactate arose by a reversible conversion of the C₄ dicarboxylic acids, to pyruvate and CO₂, *i.e.*,



In the present experiments, the lactate containing C¹³ would necessarily have to originate from succinate instead of fumarate. As previously indicated, the anaerobic oxidation of succinate to fumarate may not occur. Thus the formation of lactic acid containing C¹³ would be a definite indication of the existence of such a reaction, and also the formation of lactate by way of the C₄ dicarboxylic acids. The lactate formed on the addition of type 2 acetate did not contain C¹³ (Table I). This negative result cannot be considered conclusive because of the low yield of lactate, and also because the pyruvate formed from glucose has probably diluted the pyruvate formed from the C₄ dicarboxylic acids. This problem could possibly be settled by the addition of fumarate and malate containing C¹³ to fermentations of glucose by *Aerobacter* or *Escherichia*. The anaerobic oxidation of succinate to fumarate (if it occurs) would thus be unnecessary, and lactic acid containing C¹³ might result.

Nishina, Endo, and Nakayama (1941), with the aid of C¹⁴O₂, have shown a conversion of ethyl alcohol to radioactive malic acid by *E. coli*. Such a conversion may involve C₂ condensation to succinate. By oxidation of the succinate to oxalacetate, C¹⁴O₂ may enter the latter molecule by exchange, and the oxalacetate containing C¹⁴O₂ then be reduced to malate.

The aerobic oxidation of pyruvic acid has long been a problem to cell

physiologists. In animal tissues such as brain and liver, and in bacteria such as the lactic acid bacteria and gonococci, acetic acid has been found as an oxidation product of pyruvic acid. This is especially true when injured cells or cell-free enzyme systems were employed. Such results indicate that acetic acid or a similar C_2 compound is an intermediate in the oxidation process. The main problem thus resolves itself into an oxidation of acetic acid to carbon dioxide and water. Oppenheimer and Stern (1939) state, "The decisive key reaction consisting in the dehydrogenation of acetic acid to succinic acid has long been a theoretical postulate." This postulate involves the condensation of 2 molecules of acetic acid to succinic acid, followed by oxidation of the latter acid to pyruvic acid and CO_2 . Thus one molecule of pyruvate has been completely oxidized and the other has been recovered.

Thus direct proof has been obtained for the condensation of C_2 compounds originating from acetic acid to a C_4 compound which is isolated as succinic acid. Inasmuch as the initial compound is *acetic acid* and the end product is *succinic acid*, the reaction in question involves acetic acid condensation. Thus these results present the first positive evidence obtained for this reaction, with the implication that the reaction may function as a part of an oxidation cycle in bacteria and animal tissues.

Conversion of Acetic Acid to 2,3-Butylene Glycol

Reynolds, Jacobsson, and Werkman (1937) have shown that the addition of acetic acid to fermentations of glucose by *Aerobacter* results in an almost quantitative increase in 2,3-butyleneglycol. The suggestion was made that the added acetic acid was reduced and condensed to the glycol. The results to be presented support their suggestion in that the glycol does arise in part from acetic acid. It is doubtful, however, whether the greater portion of the glycol is formed in this manner. In other words, the conversion of acetate to 2,3-butyleneglycol probably is not an essential reaction in the formation of the glycol.

For many years acetaldehyde has been considered an intermediate in the formation of acetyl methyl carbinol by yeast. Neuberg and Kobel (1925) suggested that one molecule of synthetic acetaldehyde condenses with one molecule of nascent acetaldehyde formed by yeast preparations from glucose or pyruvic acid to form the carbinol. Dirscherl (1931) suggested the possibility of acetaldehyde coupling with pyruvic acid prior to decarboxylation to the carbinol.

On the other hand, little work has been performed on the formation of acetyl methyl carbinol and 2,3-butylene glycol by bacteria. In view of the results of Reynolds, *et al.* (1937), Mickelson and Werkman (1939) attempted to obtain evidence of the conversion of fatty acids and aldehydes to the corresponding glycols during the fermentation of glucose by *Aerobacter*. On the addition of acetaldehyde, increases in acetyl methyl carbinol, ethyl alcohol and acetic acid were obtained. However, the organism was not able to synthesize the homologous glycol or carbinol on addition of propionaldehyde and butyraldehyde. The latter compounds were reduced to the corresponding alcohols with a decrease in the yield of ethyl alcohol. Added propionic acid was reduced to the alcohol and caused an increase in the yield of 2,3-butylene glycol. It was suggested that added acetic acid was assimilated in a similar manner, without itself being transformed to the glycol. It was also suggested that perhaps synthetic acetaldehyde was condensed with some intermediate other than acetaldehyde during the formation of the carbinol.

Hammer (1936) found increases in the yield of acetyl methyl carbinol on the addition of homologues of acetaldehyde to cultures of *Streptococcus liquefaciens*. Homologues of acetyl methyl carbinol were not formed.

Silverman and Werkman (1941) were not able to show the participation of acetaldehyde in the formation of acetyl methyl carbinol by a *cell-free enzyme preparation* of *Aerobacter*. Recently, Green, *et al.* (1942) demonstrated large increases in the carbinol upon the addition of acetaldehyde to pyruvic acid in the presence of animal and yeast enzymes. The proposal was made that a condensation product of pyruvic acid and acetaldehyde was involved in the formation of the carbinol.

Thus little definite evidence exists for the occurrence of acetaldehyde as an intermediate in the formation of acetyl methyl carbinol and 2,3-butylene glycol by bacteria. The results of the present investigation prove that a C_2 compound, acetaldehyde or a closely related derivative formed by a reduction of acetic acid, is involved in the synthesis of 2,3-butylene glycol. It is also shown that the carbon to carbon linkage created in the condensation reaction, involves the carboxyl carbon atom of acetic acid. These results present the most direct evidence for the occurrence of acetaldehyde as an intermediate in the formation of 2,3-butylene glycol by intact cells of *Aerobacter*.

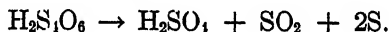
The addition of type 1 acetate (2.39 per cent C^{13}) to fermentations of glucose by *Aerobacter* results in the formation of 2,3-butylene glycol

containing 1.21 per cent C^{13} (Table I). According to a condensation reaction involving two molecules of acetaldehyde or a molecule of acetaldehyde and a molecule of pyruvic acid, the glycol formed in the above experiment should contain C^{13} exclusively in the hydroxyl carbon atoms.

To determine the position of the C^{13} , the degradation of the glycol was carried out by a preliminary oxidation to acetaldehyde by periodic acid (Brockmann and Werkman, 1933). The aldehyde was collected in ice-cold water, and the iodoform reaction performed as in the determination of acetone (Goodwin, 1920). The reaction is as follows:



Iodoform originates from the methyl carbon atoms and formic acid from the hydroxyl carbon atoms of the glycol. The residue remaining after filtration of the iodoform, was acidified and the formic acid recovered by distillation. However, it was found that hyposulfurous acid, which originates in the titration of iodine by thiosulfate, decomposed during the distillation, probably according to the following reaction:



Thus, the distillate was filtered to remove sulfur and refluxed 4 hours to remove sulfur dioxide, made alkaline, evaporated to a small volume, acidified and redistilled. The formic acid was oxidized with mercuric oxide according to Osburn, Wood, and Werkman (1933).

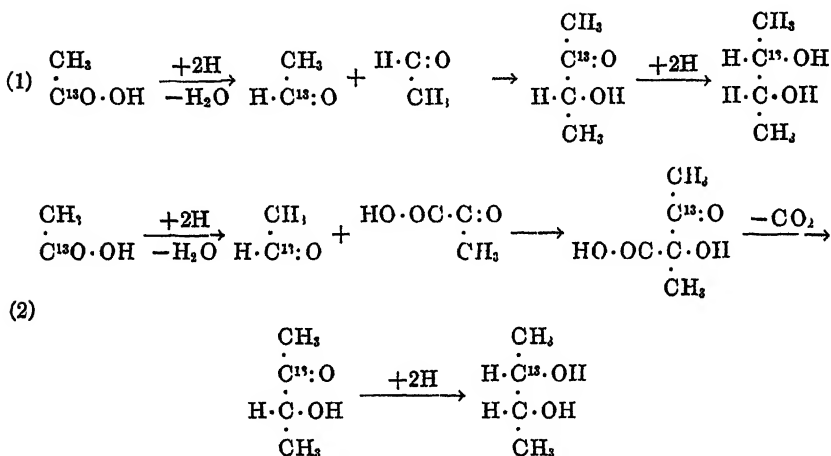
The hydroxyl carbon atoms contained 1.27 per cent C^{13} (Table II), whereas the methyl carbon atoms were normal. This result proves that acetic acid took part in a condensation reaction, with the creation of a carbon to carbon linkage, of which at least one of the carbon atoms was originally the carboxyl carbon atom of acetic acid. Calculations indicate that approximately 13 per cent of the glycol was formed from acetic acid.

On the addition of type 2 acetate, 2,3-butylene glycol was formed which contained 1.24 per cent C^{13} . Likewise, according to a condensation reaction, this glycol should contain C^{13} equally distributed between the methyl and hydroxyl carbon atoms. Table II shows that the methyl carbon atoms contained 1.23 per cent and the hydroxyl carbon atoms 1.22 per cent C^{13} . Calculations indicate that approximately 7 per cent of the glycol was formed from acetic acid.

The addition of succinic acid also resulted in the formation of 2,3-butylene glycol containing C^{13} . This experiment is comparable to those

in which acetic acid was added, because of the cleavage of succinic acid to acetic acid. Calculations indicate that approximately 47 per cent of the glycol was formed from acetic acid although this value is not reliable because of the small amount of C^{13} present in the glycol.

In regard to the mechanism of formation of the glycol in these experiments, it appears that either (1) two molecules of acetaldehyde, or (2) one molecule of acetaldehyde and one molecule of pyruvic acid are involved. However, it is not possible to determine which mechanism occurred on the basis of the position of the C^{13} in the glycol. The following reactions will illustrate:



In view of the results of Green, *et al.* (1942), it is likely that in the present experiments, the 2,3-butyleneglycol was formed by a mechanism similar to that represented by equation (2) above. The C_5 compound in the equation represents a possible structure of the intermediate compound formed in the condensation reaction. The acetaldehyde necessary for this condensation was formed by the reduction of acetic acid. In the next section it will be shown that added acetic acid is reduced to ethyl alcohol by cell suspensions of *Aerobacter*, and the conclusion is reasonable that acetaldehyde is an intermediate in that reduction.

The results of Gross, Wood, and Werkman (1942) are of interest in respect to the intermediate mechanism of glycol formation. On the addition of $C^{13}H_3 \cdot C^{13}HO$ to pyruvic acid and the cell-free *Aerobacter* preparation of Silverman and Werkman (1941), it was found that little of the added aldehyde was utilized in the synthesis of acetyl methyl car-

binol. This result is in agreement with the results of the latter workers. Thus the conclusion is necessary that two mechanisms exist for the formation of acetyl methyl carbinol by the preparations of Silverman and Werkman and Green, *et al.* In the former case, the enzyme preparation did not possess the ability to activate the added acetaldehyde or to convert it to the necessary intermediate form, whereas in the latter case this function occurs just as in the intact bacterial cell. Thus, it is evident that it is necessary to use caution in correlating results obtained with intact cells with those obtained with cell-free preparations. It can be safely stated that a reaction which occurs in a cell-free preparation will probably occur in the intact cell, but the reverse will not always be true because the enzyme may have been destroyed in the extraction procedure.

The present results prove that a C_2 compound, acetaldehyde or a closely related derivative formed by a reduction of acetic acid, is involved in the synthesis of 2,3-butylene glycol. The carbon to carbon linkage created in the condensation reaction, involves the carbon atom of acetic acid originally present in the carboxyl group.

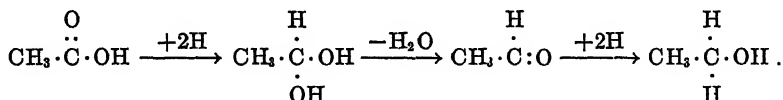
Reduction of Acetic Acid to Ethyl Alcohol

The reduction of aliphatic acids to the corresponding alcohols has been postulated for several genera of heterotrophic bacteria (Osburn, Brown, and Werkman, 1938; Mickelson and Werkman, 1939). The latter authors added propionic acid to fermentations of glucose by *Aerobacter* and found a formation of propyl alcohol. The alcohol was very likely formed by a reduction of the corresponding acid. A similar reaction was suggested in the case of acetic acid. Wood, *et al.* (1941) have presented evidence which indicates that *Propionibacterium pentosaceum* is able to reduce propionic acid to the alcohol. In a fermentation of glycerol to which $C^{13}O_2$ was added, both the propionic acid and the propyl alcohol formed contained fixed C^{13} . Inasmuch as propionic acid has been shown by the latter authors to arise by a decarboxylation of succinic acid, it is reasonable to suppose that the propyl alcohol was formed by a reduction of the propionic acid.

Ethyl alcohol containing C^{13} was formed on the addition of acetic acid and succinic acid to fermentations of glucose by *Aerobacter* (Table I). A reduction of added type 2 acetate to ethyl alcohol should result in an equal distribution of the C^{13} between the methyl and hydroxyl carbon atoms. To determine the position of the C^{13} in the alcohol, the latter

was distilled from the fermented medium and oxidized to acetic acid with potassium dichromate. The acetic acid was then degraded by dry distillation of the barium salt (cf. Slade, *et al.*, 1942). The alcohol formed on the addition of type 2 acetate contained 2.19 per cent C^{13} , and after degradation, the methyl carbon atoms were found to contain 2.16 per cent C^{13} and the hydroxyl carbon atoms 2.17 per cent C^{13} (Table II).

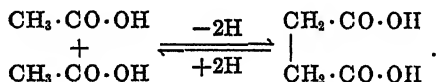
These results prove a reduction of acetic acid to ethyl alcohol by *Aerobacter*. The alcohol could not have arisen by oxidation of succinic acid because it contains a greater percentage of C^{13} than the percentage of C^{13} in the final succinic acid. The reduction may be pictured as follows:



Calculations made on the basis of the C^{13} content of the final acetic acid, indicate that on the addition of type 1 acetate, 60 per cent of the alcohol was formed from acetic acid, on the addition of type 2 acetate, 51 per cent of the alcohol was formed from acetic acid; and on the addition of succinic acid, 69 per cent of the alcohol was formed from acetic acid. Thus the reduction of acetic acid to ethyl alcohol is a reaction possessing quantitative significance, *i.e.*, under the existing experimental conditions.

SUMMARY AND CONCLUSIONS

Cell suspensions of *Aerobacter indologenes* in the presence of glucose have been shown to condense acetic acid, containing C^{13} as a tracer, to succinic acid. The carbon to carbon linkage created in the condensation involves the carbon atom originally present in the *methyl* group of acetic acid. The addition of succinic acid resulted in the reverse reaction, *i.e.*, cleavage to acetic acid. The general reaction may be represented as follows:



These results present the first positive proof of the occurrence of such a reaction in either bacteria or animal tissues.

In addition, acetic acid is reduced and condensed to 2,3-butylene

glycol. Acetaldehyde or a closely related derivative formed from acetic acid is involved in the synthesis of the glycol. The carbon to carbon linkage created in the synthesis involves the carbon atom originally present in the *carboxyl* group of acetic acid. The results constitute the most direct evidence for the participation of acetaldehyde as an intermediate in the formation of 2,3-butylene glycol. Mechanism of the reaction is discussed.

Acetic acid is also reduced to ethyl alcohol.

Calculations indicate the reactions are quantitatively significant.

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The Anaerobic Dissimilation of Pyruvate by a Cell-free Extract of *Escherichia coli*

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INTRODUCTION

In a preliminary report (Kalnitsky and Werkman, 1942) a cell-free enzyme preparation from *Escherichia coli* was described which attacks pyruvate anaerobically to produce CO₂, acetic, formic, lactic, and succinic acids. In that report it was shown that CO₂ was utilized, and in the presence of C¹³O₂, a significant amount of heavy carbon was located in the carboxyl groups of the succinic acid isolated. This paper will present the general properties of the preparation in the anaerobic dissimilation of pyruvate. It is felt that a knowledge of this mechanism and of the enzymes present will lead to a clearer understanding of the CO₂-fixation process.

Previous investigations on pyruvate with cell-free enzyme preparations from bacteria have been largely limited to studies of the aerobic dissimilation. More recently, Silverman and Werkman (1942) and Koepsell and Johnson (1942) have investigated the anaerobic dissimilation of pyruvate with cell-free preparations. Silverman and Werkman (1942) described an enzyme preparation from *Aerobacter* which converted pyruvate into acetyl methyl carbinol and CO₂. Koepsell and Johnson (1942) described an enzyme preparation obtained from *Clostridium butylicum* which converted pyruvate into acetic acid, CO₂ and molecular hydrogen. The aerobic investigations have been done chiefly by Lipmann (1939 for summary) and Still (1941). Lipmann obtained extracts of *Lactobacillus delbrückii* which oxidized pyruvate to acetate and CO₂; and Still, using the Booth-Green mill, obtained a similar pyruvic dehydrogenase system from *E. coli*. Both preparations, however, had almost no activity under anaerobic conditions.

The preparation reported here, in addition to the pyruvate enzyme, contains very active formic dehydrogenase and hydrogenase activity.

METHODS

Escherichia coli was grown in ten liters of a medium containing 0.4 per cent beef extract, 0.4 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent NaCl, 10 per cent tap water, plus distilled water to volume. The inoculated medium was continuously aerated for 24 hours at 30°C.; octadecyl alcohol was added to prevent excessive foaming. The cells were harvested by use of a Sharples supercentrifuge. The yield was between 10 and 25 g. of cells per ten liters of medium. Attempts to increase the yield failed. However, it was found that the nutrients in the medium were not exhausted after growth had taken place, for on re-sterilization and re-inoculation a second crop of cells of approximately the same weight as the first was harvested.

The enzyme preparation was obtained by mixing the wet mass of cells with clean, powdered pyrex glass according to Wiggert, *et al.* (1940) and grinding according to the modifications described by Utter and Werkman (1942). The ground cell-glass mixture was then extracted with 0.2 *M* phosphate buffer, pH 6.88, or, in a few experiments, with distilled water. Preparations obtained from cells grown on the solid agar medium of Krebs (1937) had only weak activity, whereas organisms grown in liquid culture seemed less resistant to grinding and consistently yielded stronger enzyme preparations.

Experiments were set up in Warburg cups when only pyruvate and CO₂ were to be determined. When complete fermentation analyses were performed, the experiments were carried out in 300 ml. Erlenmeyer flasks containing 70 ml. of reaction mixture, both at 30.4°C.

Pyruvic acid was determined colorimetrically by the salicylaldehyde method of Straub (1936). To determine residual pyruvate, the fermentation mixture was acidified with sulfuric or trichloroacetic acid, diluted to volume, the proteins filtered off, and an aliquot taken for the test. The Klett-Summerson photoelectric colorimeter (470 μ filter) was used. Reagent controls and known pyruvate samples were run with each test. The pyruvic acid used was freshly distilled and was kept in the crystalline form under refrigeration. Solutions of sodium pyruvate were kept frozen when not in use. Pyruvate kept in this way for several months was 80-90 per cent utilized by the enzyme. The yield of sodium pyruvate when prepared according to Peters (1938) was low but was completely utilized; whereas with the method of Robertson (1942) sodium pyruvate was easily prepared, but the enzyme system utilized this sodium pyruvate only to a very limited extent.

Carbon dioxide was collected by acidifying and aerating the fermentation mixture into an alkali tower containing 20 ml. of 1.5 *N* alkali. An aliquot of the alkali was acidified and the released carbon dioxide determined manometrically, or absorbed in ascarite and determined gravimetrically.

Volatile acids were collected by acid steam distillation. Formic acid was determined by the mercuric chloride method of Auerbach and Zeglin (1922) or by oxidation to carbon dioxide with mercuric oxide (Osburn, *et al.*, 1933). Acetic acid was determined by titration and by oxidation with persulfate (Osburn and

Werkman, 1932) after identification by the partition method of Osburn, *et al.* (1933).

The residue of the steam distillation was extracted with ether, and succinic acid was determined either by precipitation as the silver salt (Wood, *et al.*, 1942) or with succinic dehydrogenase preparations from beef heart (Krebs, 1937; using the homogenizer described by Potter and Elvehjem, 1936). Lactic acid was determined according to the method of Friedemann and Kendall (1929).

Dialysis was carried out in a collodion bag against distilled water at 0°C. C'ocarbonylase was kindly provided by Merck and Co., and flavin-adenine-dinucleotide was graciously supplied by Dr. Fritz Lipmann.

EXPERIMENTAL

1. Effect of Pyruvate Concentration

The enzyme is active on pyruvate anaerobically and liberates a large amount of carbon dioxide in a bicarbonate buffer. More than 1000 μ l. of carbon dioxide are evolved in one hour by 0.8 ml. of the enzyme; the values depend on the amount of pyruvate present, which is never completely fermented, even in low concentrations. The limiting factor is the biological availability of the substrate. The enzyme system is easily saturated since concentrations of pyruvate beyond 0.03 *M* have no effect on the rate of CO₂ evolved.

2. Effect of pH on Enzyme Activity

The ground cells were extracted with water, and phosphate buffer of a definite pH was added to each cup. After two hours the contents were deproteinized with trichloroacetic acid and diluted to a convenient volume. Residual pyruvate was determined on an aliquot containing less than 0.5 mg. per ml. The dissimilation of pyruvate (Table II) proceeds within a range of approximately pH 5.8 to 8.0; the rate shows a marked change with pH between pH 6.2 and 7.2 with the optimum near pH 6.8. In contrast to these results, the optimum pH of the aerobic mechanism of *E. coli* studied by Still was 6.17, with practically no activity at pH 7.0. The optimum for the aerobic mechanism of *L. delbrückii* (Lipmann, 1939) was between 6.0 and 6.5, and that for *Cl. butylicum* (Koepsell and Johnson, 1942) was 6.5. The preparation used in this investigation retains almost 50 percent of its activity at pH 7.2, whereas below 6.2 and above 7.2 there is a sharp decrease in the pyruvate utilized.

3. Effect of Heat Treatment on Activity of the Enzyme Preparation

The preparation is not affected by temperatures up to 40°C. for five minutes (Table III). When heated at 43°C. for five minutes, it becomes

TABLE I

Dissimilation of Pyruvate by Enzyme Preparation of E. coli

Cup no.	Pyruvate concentration (M)	Per cent fermented	μ l. CO ₂ evolved	
			1 hr.	15 min.
1	0.015	90.1	301	256
2	0.030	91.7	530	318
3	0.045	91.2	812	334
4	0.060	85.7	828	
5	0.075	70.1	951	320
6	0.090	63.9	1076	359

Each cup contained enzyme, 0.8 ml.; NaHCO₃, 0.045M (No. 5 and 6, 0.06M NaHCO₃); pyruvate, in indicated concentrations. Total volume, 2 ml. Atmosphere, 10 per cent CO₂ in N₂.

TABLE II

Effect of pH on the Dissimilation of Pyruvate by the Enzyme Preparation of E. coli

pH	5.4	5.8	6.2	6.4	6.77	6.94	7.2	7.3	7.5	7.9
Pyruvate fermented (mg.)	0.0	0.42	3.38	4.01	4.33	3.96	2.37	0.90	0.85	0.42
Per cent pyruvate fermented	0	8	64	76	82	75	44	17	16	8

Each cup contained enzyme, 0.6 ml.; pyruvate, 5.28 mg./2 ml. (0.03M); phosphate buffer (0.25M). Total volume, 2 ml. Time, 2 hrs. Atmosphere, N₂.

TABLE III

Effect of Temperature on Activity of the Enzyme Preparation

Temperature	Pyruvate (initial 10.56 mg.)	CO ₂ evolved μ l.	Inactivation per cent
	5 min. fermented mg.		
30°C.	8.99	729	0
40°C.	8.99	714	0
43°C.	9.00	746	0
47°C.	9.09	690	0
50°C.	8.46	594	0.2
52°C.	7.49	533	17
53°C.	4.18	332	54
55°C.	1.19	—49	87
57°C.	0.00	—56	100
Control (no enzyme)	0.00	—1	

Enzyme heated at temperature indicated for 5 minutes. Each cup contained enzyme, 0.8 ml.; pyruvate, 10.56 mg. (0.06M); NaHCO₃ (0.045M). Total volume in each cup, 2.0 ml. Atmosphere, 10 per cent CO₂ in H₂. Time, 1 hr.

translucent, but no inactivation occurs. At 47°C. some of the proteins precipitate, and the solution becomes opaque; but no loss in activity occurs, as judged by the pyruvate utilized. At 50°C. the preparation still retains the greater part of its activity, and inactivation is only 6 per cent. From 50° to 55°C. inactivation gradually increases to 87 per cent, and between 55° and 57°C. activity is completely destroyed.

The preparation obtained by Silverman and Werkman (1941) from *Aerobacter* retained 6 to 7 per cent of its activity on pyruvate when heated for five minutes at 65°C. This stability correlates with the results of Melnick and Stern (1940) who found that five minutes at 60°C. reduced the activity of yeast carboxylase to 4 per cent. The present preparation is less stable to heat than those obtained from *Aerobacter* and yeast and is comparable to Still's aerobic pyruvic dehydrogenase preparation which is inactivated when heated to 55–60°C.

4. *Effect of Drying on Activity of the Enzyme Preparation*

The clear liquid was tested for activity, then frozen and dried overnight in a vacuum desiccator. The dried material was then ground gently with a mortar and pestle and resuspended in its original volume of distilled water. There was no loss of activity in preparations tested after a few days. For example, 778 μ l. CO₂ were liberated by the liquid in one hour, and 799 μ l. CO₂ by the dried preparation. One batch of dried material was allowed to remain at 5°C. for 53 days with a resulting sharp decrease in activity as tested manometrically (99 μ l.). When the preparation was dried without first being frozen, it was completely inactivated.

5. *Formic Dehydrogenase and Hydrogenase Activity*

The enzyme preparation contains very strong formic dehydrogenase activity and reduces methylene blue in less than two minutes with formic acid as the hydrogen donator (Table IV). Stickland (1929) obtained a cell-free enzyme preparation from *E. coli* through tryptic digestion, containing the enzyme, formic dehydrogenase. Gale (1939) by the use of the Booth-Green mill obtained this same enzyme. In neither case, however, was the active formic dehydrogenase separated from the solid particles. The enzyme preparation to be described is a clear liquid, and was obtained by centrifuging off ground cells and debris in a Beams ultracentrifuge (capacity, 5 ml.) at approximately 100,000 r.p.m. for 10 minutes. The activity of the formic dehydrogenase thus does not seem to be bound to the solid particles.

The enzyme hydrogenase, which reduces methylene blue with gaseous hydrogen as the only hydrogen donator, is also present in a very active form (Table IV). All decolorization times were taken as the time necessary for complete reduction of methylene blue.

Both these enzymes, as present in this preparation, are quite stable. They can be reduced to a powder form by freezing and drying *in vacuo*, and resuspended in water with no appreciable loss in activity. After remaining in the dried form for almost two months, the enzymes still retained the greater part of their activity (Table IV). Lee, *et al.* (1942) have obtained active hydrogenase preparations from *Azotobacter*, and Bovernick (1941) by acetone treatment of *E. coli* has obtained cell-free powders containing active hydrogenase.

TABLE IV

Formic Dehydrogenase and Hydrogenase Activity of E. coli Enzyme Preparation

Enzyme	None	Treatment	Controls (no substrate)
		Dried (53 days)	
	Min. *	Min.	Min.
Formic dehydrogenase	1 75	<5	>60
Hydrogenase	1	7	>60

* To decolorize methylene blue.

Each cup contained enzyme, 0.8 ml.; NaHCO_3 , 0.045M; methylene blue (1/14,000). Substrate for formic dehydrogenase was 0.5 ml. 0.1M HCOOH . Atmosphere, N_2 . Substrate for hydrogenase, H_2 . Total volume, 2.8 ml.

Aeration during growth destroys formic hydrogenlyase (Yudkin, 1932). Bacteria used in the present experiments were grown under aeration and did not contain that enzyme; *i.e.*, formic acid was not broken down to H_2 and CO_2 , even after 24 hours' incubation. The fact that both formic dehydrogenase and hydrogenase are present, but not formic hydrogenlyase, disposes of the possibility that the absence of formic hydrogenlyase may be due to separation of these two enzymes within the intact cell.

6. Activity on Fumarate and Oxalacetate

In view of the strong hydrogenase activity of the preparation, the suitability of compounds other than methylene blue as hydrogen acceptors was determined. Acetaldehyde was unsuitable, however. Strong activity was exhibited with fumarate and oxalacetate as accep-

tors of gaseous hydrogen (Table V). This is of interest in view of the postulated occurrence of these two compounds in the formation of succinic acid from pyruvate. The reduction of oxalacetate is much slower than that of fumarate. However, oxalacetate is rapidly decarboxylated, a fact which suggests a similarity between this system and the acetone preparation of *Micrococcus lysodeikticus* reported by Krampitz and Werkman (1941).

TABLE V
Activity of Enzyme Preparation on Fumarate and Oxalacetate

	CO ₂ evolved (μ l.)	H ₂ uptake (μ l.)
Fumarate.....	{ (-472) 0 (-490) 0	-428 -557
	+413	-96
Oxalacetate	{ +443 +446 +412	-92 -106 -112
Oxalacetate (spontaneous)	{ +87 +84	-4

Figures not corrected for spontaneous action of oxalacetate.

Substrate concentration, 0.02M Enzyme, 0.8 ml. (In oxalacetate control, 0.8 ml. phosphate (0.2M; pH, 6.88) added instead of juice.) Total cup contents, 2.0 ml. Atmosphere, H₂. Time, 1 hr.

7. Products of Pyruvate Dissimilation

Pyruvate is attacked anaerobically to form CO₂, acetic, formic and succinic acids, and a trace of lactic acid. No H₂ is formed. Aerobically, oxygen is not taken up, although the pyruvate is fermented. Under an atmosphere of N₂ and with no bicarbonate present in the medium, very little succinic acid is formed. The effect of different gaseous atmospheres on succinate formation was investigated, and it was found that the yield of succinate was highest under an atmosphere of 5-10 per cent CO₂ in H₂. This gas mixture was obtained in Experiments 2, 3 and 4 (Table VI) by the addition of acid phosphate to a medium which contained bicarbonate and was under an atmosphere of H₂. Experiment 1 was carried out in a large cup on the respirometer, whereas the others were carried out in a stationary 300 ml. Erlenmeyer flask connected to a NaOH bead tower. The fermentation was stopped by addition of sul-

furic or metaphosphoric acid. Carbon balances and O/R indices were determined in order to check the accuracy of the analyses. A small error in the determination of a strongly oxidized product (for example CO_2) will result in a large error in the O/R index, which is the case in Experiment 2.

TABLE VI

Products of Pyruvate Dissimilation by E. coli Enzyme Preparation

Experiment no.	1	2	3	4
Pyruvate fermented (mM)	2.59	2.52	2.85	2.67
Products per 100 mM of pyruvate fermented				
CO_2 (mM)	5.65	5.2	7.4	6.38
Formic acid (mM)	76.81	62.0	80.7	69.3
Acetic acid (mM)	88.62	74.4	80.7	76.1
Lactic acid (mM)	3.32	2.8	2.5	3.75
Succinic acid (mM)	4.71	12.8	8.4	14.36
Carbon recovery, per cent	96	92	96	99
O/R index	0.93	0.84	1.03	0.97

Atmosphere

N_2 CO_2 in H_2 CO_2 in H_2 CO_2 in H_2

Experiment no. 1—contained juice, 18 ml.; pyruvate (0.12M). Total volume, 30 ml. Time, 2 hrs.

Experiment no. 2—contained juice, 23 ml.; pyruvate (0.04M); NaHCO_3 (0.04M); phosphate, 5 ml. (0.1M; pH, 6.2). Total volume, 70 ml. Time, 4 hrs.

Experiment no. 3—contained juice, 30 ml.; pyruvate (0.05M); NaHCO_3 (0.05M), phosphate, 7 ml. (0.1M). Total volume, 75 ml. Time, 4 hrs.

Experiment no. 4—contained juice, 38 ml.; pyruvate (0.04M); NaHCO_3 (0.07M); phosphate, 10 ml. Total volume, 75 ml. Time, 4 hrs.

8. Components of the Enzyme System

The enzyme system is easily inactivated by dialysis for half an hour and reactivated on the addition of phosphate (Table VII). The necessity of phosphate in the oxidation of pyruvate has been shown by Virtanen and Karström (1931), Lipmann (1939), Banga, *et al.* (1939) and Still (1941). Silverman and Werkman (1941) showed phosphate was necessary for the anaerobic conversion of pyruvate to acetyl methyl carbinol, and Koepsell and Johnson (1942) also demonstrated the necessity of phosphate in the evolution of H_2 from pyruvate anaerobically. Thus, phosphate is also necessary for the breakdown of pyruvate to acetic and formic acids *via* the hydroclastic reaction. Concentrations of phosphate up to 0.01M (Table VIII) have no appreciable effect on the

dissimilation of pyruvate. There is only a slight effect when the phosphate concentration is increased to $0.015M$, and a decided effect at $0.018M$, above which more phosphate does not appreciably affect the pyruvate breakdown. These results agree with those of Lipmann, and Koepsell and Johnson, who reported optimum phosphate concentrations of $0.015M$ and $0.02M$ for pyruvate breakdown; below these concentrations, the rates of the two systems were proportional to the phosphate concentrations.

TABLE VII

*Effect of Phosphate on Pyruvate Dissimilation by Dialyzed *E. coli* Juice*

Experiment No.	Dialyzing time (min.)	μ l. CO_2 evolved, 1 hr. Dialyzed juice	Dialyzed juice + PO^{--}
1	25	203	745
2	30	147	586
3	35	0	560
4	45	109	530
5	90	17	21

Each cup contained dialyzed juice, 1.0 ml; pyruvate ($0.045M$); $NaHCO_3$ ($0.045M$); phosphate ($0.02M$; pH, 6.88) + water to 2.0 ml. Atmosphere, 10 per cent CO_2 in H_2 . Time, 1 hr.

TABLE VIII

*Effect of Phosphate Concentration on Pyruvate Dissimilation by Dialyzed *E. coli* Juice*

Phosphate (M)	—	0	003	0	005	0	008	0	01	0	013	0	015	0.018	0	02	0	025
μ l. CO_2 evolved	11	17	26	61	32	107	89	507	434	536								

Juice dialyzed 37 minutes. Each cup contained dialyzed juice, 1.0 ml.; pyruvate ($0.045M$); $NaHCO_3$ ($0.045M$) + phosphate in indicated concentrations. Total volume of each cup, 2.0 ml. Atmosphere, 10 per cent CO_2 in H_2 . Time, 2.5 hrs.

In order to demonstrate the necessity of manganese and cocarboxylase in the system, the enzyme preparation had to undergo extensive dialysis, during which it lost a large part of its activity. After 100 minutes' dialysis, the addition of phosphate and cocarboxylase markedly stimulated the reaction, whereas the addition of Mn had a somewhat smaller effect (Table IX). The necessity of Mn^{++} and cocarboxylase in anaerobic pyruvate breakdown correlates with their requirement in the aerobic oxidation of pyruvate (Ochoa, 1939; Lipmann, 1939; and Still, 1941).

Mg^{++} was not as effective as Mn^{++} during the first hour (Table X). However, at the end of three hours, the effects of Mn^{++} and Mg^{++} were quite similar.

Lipmann (1939) reported flavin-adenine-dinucleotide to be a component in the dismutation of pyruvate by extracts of *L. delbrückii* and

TABLE IX
*Effect of Phosphate, Cocarboxylase and Manganese on Pyruvate
Dissimilation by Dialyzed Juice of E. coli*

	Additions to manometer cups (ml.)				
Juice.....	1.0	1.0	1.0	1.0	1.0
Pyruvate (0.3M).....	0.3	0.3	0.3	0.3	0.3
NaHCO ₃ (0.3M).....	0.3	0.3	0.3	0.3	0.3
Phosphate (0.2M; pH, 6.88).....		0.2		0.2	0.2
Cocarboxylase (150 µg./ml.).....		0.1	0.1		0.1
Mn (0.05M).....		0.1	0.1	0.1	
H ₂ O.....	0.4		0.2	0.1	0.1
µl. CO ₂ , 1 hr.....	-14	104	-14	16	54

Juice dialyzed 100 minutes.

Atmosphere, 10 per cent CO₂ in H₂.

TABLE X
*Comparison of Mn and Mg Effects on Pyruvate Dissimilation by Dialyzed E. coli
Juice*

Cup contents	1 hr.	2 hrs.	3 hrs.
Dialyzed juice + cocarboxylase, PO ₄ ⁻⁻⁻ , Mn.....	645	1123	1281
Dialyzed juice + cocarboxylase, PO ₄ ⁻⁻⁻ , Mg.....	37	325	1054
Dialyzed juice.....	-37	40	160

Juice dialyzed 36 minutes.

Cups contained dialyzed juice, 1.0 ml.; pyruvate (0.045M); NaHCO₃ (0.045M); phosphate buffer (0.02M; pH, 6.88); cocarboxylase, 15 µg.; Mn or Mg (0.005M). Total volume, 2.3 ml. Atmosphere, 10 per cent CO₂ in H₂.

Still (1941) found slight increases in pyruvate oxidation by *E. coli* juice on addition of the flavin compound. Under the conditions of our experiments, addition of flavin-adenine-dinucleotide was not effective.

After dialysis for one and one-half to two hours, the pyruvate system is inactivated, and activity is not restored on addition of phosphate, Mn^{++} and cocarboxylase. Further addition of co-enzyme 1, adenylic acid, adenosinetriphosphate, Mg^{++} , riboflavin, fumarate, Ca panto-

thenate or biotin, in various combinations failed to restore the activity of the dialyzed enzyme preparation, whereas the addition of boiled yeast juice gave a stimulation. It is possible that some other component, besides phosphate, Mn^{++} , cocarboxylase and protein, is a part of this system. A pronounced dilution effect of the system after dialysis was also noticed.

SUMMARY

1. An active, cell-free extract has been obtained from *E. coli*, which attacks pyruvate anaerobically, producing acetic, formic, lactic and succinic acids, and CO_2 .

2. The enzyme system is active within a pH range of 6.2–7.0 and can be dried *in vacuo* without any immediate loss of activity. The dried preparation slowly deteriorates with age.

3. The preparation contains very strong formic dehydrogenase and hydrogenase activity. Formic dehydrogenase does not seem to be associated with solid particles. Both enzyme systems are quite stable and can be converted to a dry powder, in which form they retain their activity for some time.

4. Fumarate and oxalacetate are reduced with gaseous H_2 , whereas acetaldehyde is not. In addition, the enzyme system exhibits strong decarboxylating activity on oxalacetate.

5. Inorganic phosphate, Mn^{++} and cocarboxylase were shown to be components of the enzyme system in the anaerobic dissimilation of pyruvate. The optimum concentration of phosphate was 0.018*M*. At higher concentrations no further increase in the rate of dissimilation was observed.

6. Dialysis for 1½ to 2 hours inactivates the system. Addition of various compounds, alone or in combination, did not restore the activity, whereas the addition of boiled yeast juice had some effect.

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ERRATA

Vol. 1 p. 55 Table III heading:

read *per 10 g. Dry Milk* for *per 10 cc. Milk*

p. 56 Table IV heading:

read *per 10 g. Dry Milk* for *per 10 cc. Milk*

read *Micrograms fluorine per 10 g. dry milk* for *Micrograms fluorine per 10 cc. milk*

The Significance of the Amino Groups for the Oxidation of Various Compounds by the Cholera Vibrio (*V. Comma*)

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INTRODUCTION

Recent work has suggested that vitamins may act as prosthetic groups for certain enzymes in the bacterial as well as in the mammalian cell. The action of certain of the newer drugs, for instance sulfanilamide, may be explained by their ability to displace, because of their related structure, the prosthetic group from the specific protein in the cell. The action of the older drugs, as for example formaldehyde, mercury salts, etc., cannot be explained on this basis. It seems probable that these drugs react directly with some special groups on the protein of the bacteria so that it can no longer behave as a catalyst for metabolic reactions. Such groups may be sulfhydryl, amino, carboxyl, aldehyde, or ketone groups. The integrity of the sulfhydryl group on the protein has been shown to be essential for the activity of certain enzymes of mammalian cells, and the effect of copper on them can be explained by its direct action on this group. It is the purpose of this work to present certain facts which indicate the importance of another group, namely, the amino group, for the catalytic activity of certain bacteria. *Vibrio comma* and *Escherichia coli* were chosen for comparison because they are not capsulated, they both grow in the human intestine, and they can be grown on identical media.

EXPERIMENTAL

An avirulent strain of *V. comma* was obtained from Dr. N. R. Ziegler of the University of Missouri. It was grown on agar slants containing beef extract for 24 hours at 37°C. The bacteria were then washed off with 50 cc. of distilled water and centrifuged. The centrifuged bacteria

were suspended in 0.05 *M* phosphate buffer at pH 7.8 so that 1.0 cc. contained approximately 4.0 mg. dry weight. 0.5 cc. of the suspension was used in each Warburg vessel which contained a final volume of 2.0 cc. *E. coli* was grown and prepared in exactly the same way.

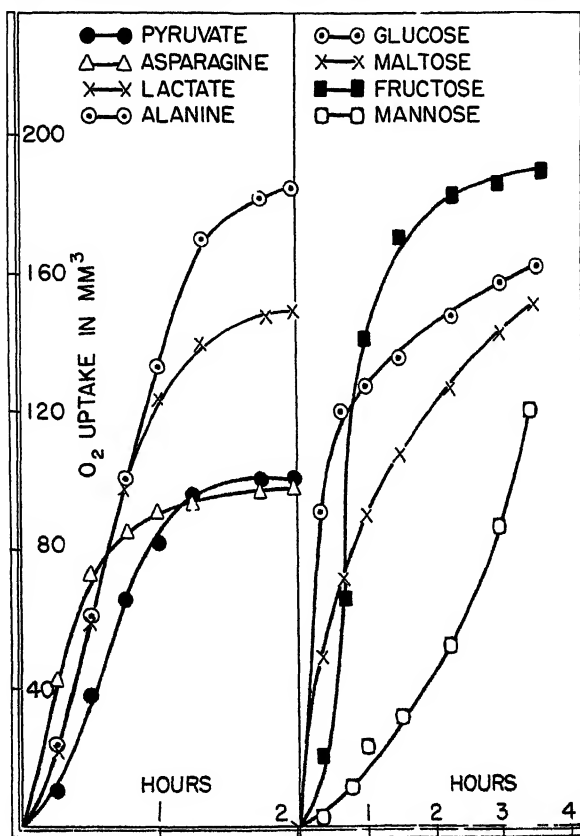


FIG. 1

The Oxidation of 0.4 mg. of Each of the Indicated Compounds by *Vibrio comma* at pH 7.8 and 37° C.

The oxygen taken up by the suspension was measured by the readings of the manometer after the carbon dioxide had been absorbed by NaOH placed in the center well of the Warburg vessel. The production of carbon dioxide was measured after the addition of acid from the side arm of the vessel, to displace the carbon dioxide in solution. This was

done when the oxidation was finished. Such a preparation of *V. comma* can be shaken for several hours in a Warburg vessel, and only very small amounts of oxygen are used up, or carbon dioxide produced, unless a suitable substrate is also present. Fig. 1 shows that this preparation oxidizes various sugars as well as lactate, pyruvate, alanine, and asparagine. Of the sugars, glucose is oxidized most rapidly followed by maltose. Both mannose and fructose show a definite latent period. This is slightly shortened but not eliminated when saline is substituted for the buffer, but this change has no effect on the other oxidations. Table I shows the amounts of oxygen taken up and the carbon dioxide produced by a number of compounds which are substrates for the enzymes of *V. comma*. The sugars take up a little more than 3 atoms of oxygen

TABLE I

The O₂ Uptake and CO₂ Production of Various Substrates in the Presence of V. comma at pH 7.8 and 37° C.

The control figures, that is, in the absence of the substrates, have been subtracted. These were very small

	Glucose	Fructose	Mannose	Maltose	Lactate	Pyruvate	Alanine	Serine	Glycine	Acetate	Asparagine	Succinate	Fumarate	Malate	Oleate
O ₂ Uptake															
atoms/mol. . .	3-4	3-4	3	3-4	3	2	4	4	2	3-4	3	6	5	4	25
CO ₂ production															
molecules/mol..	3	2-3	2-3	3	1-2	1	2	3	1-2	1-2	2	3	3	3	6

per molecule and give off 2 or 3 molecules of carbon dioxide. The relationship between alanine and pyruvic acid is complicated. The latter takes up exactly 2 atoms of oxygen and gives off 1 molecule of carbon dioxide per molecule. The end product has not been identified but it is not glycolic acid. Alanine, however, takes up 4 atoms of oxygen and gives off 2 molecules of carbon dioxide per molecule. This difference between alanine and pyruvic acid is not accounted for by the presence of small amounts of ammonia because ammonium pyruvate behaves like the sodium salt. Evidently alanine is not oxidized to pyruvic acid although lactic acid probably is.

The dicarboxylic acids are oxidized rather slowly, and the oxygen uptakes indicate that succinic acid goes through fumaric to malic which is then further oxidized. Malonic acid in ten times the concentration

has no effect on the oxidation of succinic acid. Asparagine presumably is not oxidized through succinic acid. The presence of two free carboxyl groups seems to decrease the rate of oxidation, for aspartic acid is oxidized much more slowly than asparagine. As well as the amino acids listed in the table, proline, tyrosine, phenylalanine, and glutamic acid are slowly oxidized. In all cases, deamination also occurs. Methionine, valine, leucine, phenylglycine, and the *D*-amino acids are not attacked, and negative results were also obtained with amines, hypoxanthine, sarcosine, ethyl alcohol, furfural, glycolic and formic acids. Citric acid is oxidized slowly if at all. At pH 6.7 the oxidation of all the substrates is slower than at pH 7.8, and pyruvate is affected more than glucose, lactate, asparagine, or alanine. At the end of 30 minutes the oxygen uptake of pyruvate at pH 6.7 was only 25 per cent of that at pH 7.8; of glucose, 68 per cent; of lactate, 63 per cent; of asparagine, 46 per cent; and of alanine, 41 per cent. At pH 8.2 the rate of oxidation of all the substrates is increased by about 10 per cent.

The Effect of Formaldehyde

V. comma and *E. coli* were found to oxidize glucose and pyruvate at approximately equal rates. However, the oxidations of glucose and pyruvate by *V. comma* are much more sensitive to the presence of small quantities of formaldehyde than the oxidation of these substrates by *E. coli*. Pyruvate oxidation by *V. comma* is the most sensitive to the presence of formaldehyde. This is shown in Table II. The oxidation of fructose by *V. comma* is as much inhibited by formaldehyde as pyruvate, the other sugars behave like glucose, and acetate and asparagine like succinate. The small oxygen uptake of the control which occurs without added substrate is about one half as sensitive to formaldehyde as the pyruvate oxidation. For the following experiment the oxidation of pyruvate by *V. comma* was therefore used, and $8.4 \times 10^{-4}M$ formaldehyde caused a 95 per cent inhibition. The dry weight of bacteria in each Warburg vessel was 2.4 mg., and this weight contained as estimated by the Van Slyke technic, 0.0415 mg. amino nitrogen which in 2 cc. is equivalent to $13.2 \times 10^{-4}M$ NH_2-N . Therefore when the oxidation of pyruvate is inhibited almost 100 per cent there are still free amino groups on the bacteria.

Formaldehyde combines with protein in alkaline solutions, and the complex formed is dissociated in acid. It has not been possible to acidify the bacteria sufficiently to show dissociation but it can be shown

that formaldehyde takes longer to reach a maximal inhibition at pH 6.7 than at 7.8. Thus the inhibition of pyruvate oxidation after 20 and 40 minutes by a given amount of formaldehyde at pH 7.8 was 37 per cent and 49 per cent respectively; at pH 6.7 the values were 20 per cent and 35 per cent. A maximum of 55 per cent was reached by both. This may be considered as suggesting that the inhibition is the result of the formaldehyde combining with certain amino groups.

The Effect of Nitrous Acid

The experiments done with formaldehyde were repeated under the same conditions with NaNO_2 which also reacts with NH_2 groups. Again the oxidation of pyruvate by *V. comma* was three to four times more

TABLE II

The Effect of Different Concentrations of Formaldehyde on the Oxidation of Various Substrates by V. comma and E. coli at pH 7.8 and 37° C.

The *E. coli* suspensions had one-half as many bacteria as determined by the dry weight, as the *V. comma* suspensions

Conc. of formaldehyde	<i>V. comma</i>			<i>E. coli</i>	
	Per cent Inhibition				
	Glucose	Pyruvate	Succinate	Glucose	Pyruvate
$4.2 \times 10^{-4}M$	21	44	15	—	—
$8.4 \times 10^{-4}M$	53	77	36	2	0

sensitive to nitrite than the oxidation of pyruvate by *E. coli*. These facts suggest that nitrite and formaldehyde are reacting with the same groups in *V. comma*, and that these groups are the ones responsible for the oxidation of pyruvate. Nitrite should inhibit only in acid solutions when free nitrous acid is present. This was found to be the case, for, at pH 6.7, 0.4 mg. of NaNO_2 caused a 60 per cent inhibition of the oxidation of pyruvate by *V. comma* but there was no inhibition at pH 7.8.

If 0.3 cc. of Nessler's reagent is added to a 2 cc. suspension of cholera vibrios in buffer and the mixture is allowed to stand at room temperature for 10 to 15 minutes a deep orange precipitate forms which can be centrifuged off and shown to contain the clumped cholera organisms in various stages of disintegration. Under exactly similar conditions, the same amount of *E. coli* produces a light yellow precipitate. If the

V. comma are shaken at pH 6.7 with sufficient nitrite to cause almost complete inhibition of pyruvate oxidation, the addition of Nessler's solution produces only a light yellow color similar to that of *E. coli*. If the vibrios are shaken with the same amount of nitrite at pH 7.8 the deep orange color develops as in the control. This indicates that certain free amino groups are necessary for the development of the orange color. Similar experiments could not be done with formaldehyde because it reduces Nessler's solution and thus interferes with the color. Since it has been impossible to reproduce the deep orange of the cholera protein with other proteins such as egg albumen and casein or with amino acids or thiamin it is possible that certain of the amino groups in *V. comma* have a special configuration. Diazotization of a suspension of the bacteria with the sulfanilamide reagent gave no color which indicates that the amino groups are not attached to a ring.

The Estimation of the Amino Groups of V. comma and E. coli

Thoroughly washed suspensions of *V. comma* were put into the Van Slyke apparatus, and the nitrogen evolved was measured in the usual way. The nitrous acid was allowed to react with the bacteria for varying lengths of time up to 40 minutes and it was shown that the maximal amount of nitrogen was evolved during the first 5 minutes. Protein is not hydrolyzed under these conditions, and the nitrogen must have been evolved from free amino groups. The figure obtained was 1.54 mg. of $\text{NH}_2\text{-N}$ per 100 mg. dry weight of bacteria which represents the average of four determinations of different cultures washed a different number of times. These values checked within 5 per cent. This figure was compared with that obtained with *Escherichia coli*. *E. coli* was grown on the same medium as *V. comma*, for the same length of time, and was treated in exactly the same way. The figure obtained by the Van Slyke technic for *E. coli* was 1.25 mg. of $\text{NH}_2\text{-N}$ per 100 mg. dry weight. Thus *V. comma* has 24 per cent more reactive amino groups than *E. coli*.

The Estimation of the Carboxyl Groups

Since the evidence suggests that amino groups are reactive in both *V. comma* and *E. coli*, it was of interest to attempt an investigation of the associated carboxyl groups. Accordingly washed suspensions of both bacteria (10 mg. dry weight in 5.0 cc. of water) were titrated to phenolphthalein and 1.0 cc. of a 40 per cent solution of neutralized

formaldehyde added and the mixture immediately titrated to the same end point with 0.01 *N* NaOH. The results expressed in terms of molarity (1.0 mg. dry weight/cc.) are as follows: for *V. comma*, 0.67×10^{-3} *M* COOH groups compared to 1.10×10^{-3} *M* NH₂-N groups; for *E. coli*, 0.45×10^{-3} *M* COOH groups compared to 0.89×10^{-3} *M* NH₂-N groups. Thus in *V. comma* there are almost twice the number of amino groups than carboxyl groups, and in *E. coli* just twice.

The Possible Presence of Aldehyde or Ketone Groups

In order to test whether such groups might be present in the cell, phenylhydrazine was used. As in the case of nitrite and formaldehyde, concentrations that caused effective inhibitions in *V. comma* were without effect on the oxidation of the same substrates by *E. coli*. The lowest concentrations of phenylhydrazine inhibit the oxidation of asparagine first, and pyruvate is the least sensitive to the drug. Thus with concentrations of phenylhydrazine of 3.4, 6.9, and 10.3×10^{-5} *M* the inhibition of the asparagine oxidation after one hour was 44%, 73%, and 87% respectively, whereas pyruvate was inhibited 0%, 28%, and 63%. The oxidations of the other substrates are intermediate in sensitivity between these extremes. The phenylhydrazine was added 10 to 15 minutes before the substrates. The inhibition develops fairly slowly to a maximum at an hour to an hour and a half at pH 7.8. At pH 6.7 the inhibition develops more slowly but reaches the same maximum. This is in accordance with the fact that most phenylhydrazones are formed more rapidly in alkaline solutions. The increasing inhibition with time indicates that the presence of excess pyruvate or glucose is not causing the phenylhydrazine to dissociate from the bacteria to combine with these substrates. 2.0 mg. of pyruvate or glucose were used and 0.01–0.03 mg. of phenylhydrazine. If the assumption is made that phenylhydrazine combines quantitatively with ketone or aldehyde groups then in this experiment 10.4×10^{-5} *M* phenylhydrazine was necessary to inhibit almost completely the oxidation of asparagine by 1.7 mg. dry weight of *V. comma* in 2.0 cc. containing 9.3×10^{-4} *M* NH₂-N. In other words, there at least ten times as many amino groups as ketone or aldehyde groups on the bacteria.

The Effect of Bichloride of Mercury

This salt is very toxic to *V. comma* as it is to *E. coli* and other bacteria and inhibits the oxidation of various substrates by cholera vibrios in the

same order as formaldehyde and nitrite. $0.9 \times 10^{-6} M$ $HgCl_2$ inhibits the oxidation of pyruvate 50 per cent, and $2.1 \times 10^{-6} M$ inhibits 94 per cent when 2.6 mg. dry weight of *V. comma* in 2.0 cc. containing $14.3 \times 10^{-4} M$ amino groups are used. Assuming that the mercury reacts quantitatively, then whatever group it reacts with is present in a 700 times smaller concentration than the amino groups. Mercury possibly combines with protein to form a salt with the carboxyl group, or a complex with amino or sulfhydryl group. A thick suspension of *V. comma* containing 15 mg. dry weight of bacteria failed to give a positive nitroprusside test indicating that either the sulfhydryl groups are not available or that they are present in a very low concentration.

DISCUSSION

The question arises as to the validity of conclusions drawn from figures obtained on bacteria by the Van Slyke and formol titration methods. It is obviously impossible to decide whether the reagents come in contact with all the amino and carboxyl groups or only certain ones. Any attempt to break up the cell before estimating these groups may create new reactive groups or destroy those already present, and the results would therefore have no significance. The reactive groups estimated in this work are those to which the reagents have access, and this may depend on the degree of destruction of the cell and the permeability of the cell membrane, which may be different in *V. comma* and *E. coli*. In spite of this, however, the facts suggest certain possibilities with respect to the reactive groups in these bacteria. Both bacteria contain reactive amino and carboxyl groups on their surface or in places available to the reagents. *V. comma* contains more reactive or available amino groups per milligram dry weight than *E. coli*. Nevertheless the oxidation of pyruvate and certain other substrates by *V. comma* is more sensitive to reagents such as formaldehyde and nitrous acid, which are known to react with amino groups, than the oxidation of the same substrates by *E. coli*. This indicates that there are certain amino groups in *V. comma*, which, possibly because of the configurations of the surrounding molecules, react more readily with low concentrations of formaldehyde and nitrous acid and that these amino groups are attached to enzymes involved in the oxidation of certain substrates. The conclusions drawn from the experiments with phenylhydrazine are more equivocal because this reagent reacts slowly and may therefore not only combine with ketone groups but may also produce other non-specific effects. The failure to demon-

strate sulfhydryl groups in these bacteria makes it impossible to draw any conclusions about the mechanism of action of mercury salts.

SUMMARY

1. The reactive amino and carboxyl groups in *Vibrio comma* and *Escherichia coli* have been determined. In both cases there are about twice as many amino groups as carboxyl groups. *V. comma* has 24 per cent more reactive amino groups than *E. coli*.

2. The oxidation of pyruvic acid by *V. comma* is more sensitive to low concentrations of formaldehyde and nitrite than the oxidation by *E. coli*. Nitrite inhibits only in acid solution.

3. The addition of Nessler's reagent stains the protein of *V. comma* deep orange but has no such effect on *E. coli*. After treatment with nitrous acid, this staining reaction disappears. The addition of nitrite in alkaline solutions does not affect the staining.

4. Since formaldehyde and nitrous acid both react with amino groups, and specifically inhibit the oxidation of pyruvate by *V. comma*, it is suggested that certain NH_2 groups are necessary for the enzyme catalyzing this reaction.

5. The oxidation of asparagine by *V. comma* is more sensitive to phenylhydrazine than its oxidation by *E. coli*.

6. The oxygen uptake and the carbon dioxide production has been measured for several sugars and amino acids which act as substrates for the enzymes of *V. comma*.

Production of Cysteine from Methionine by Liver Slices¹

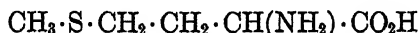
Norman F. Floyd and Grace Medes

From the Lankenau Hospital Research Institute, Philadelphia

Received February 17, 1943

INTRODUCTION

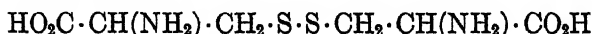
It has been demonstrated that methionine



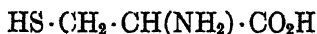
incubated with liver slices under aerobic conditions gives rise to two end products which can be determined quantitatively, *a* sulfate (1), which amounts to about two per cent of the added methionine, and *b* the deaminated methionine α -keto- γ -methiobutyric acid (2, 3)



which varies from twenty to thirty per cent of the added methionine. It has also been demonstrated by animal experiments that methionine gives rise to cystine,



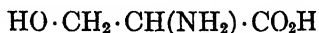
in the body (4). Among the theories put forward to account for the conversion are two which assume the ultimate transfer of the sulfur to another carbon chain. Brand (5) postulates a reaction between methionine or homocysteine and amino acrylic acid with the formation of a complex intermediate and a final splitting of the molecule with the sulfur attached to the three-carbon portion in the form of cysteine



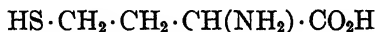
Toennies (6) calls attention to the possible formation of a sulfonium

¹ Aided by a grant from Mrs. L. Elizabeth Nax.

intermediate by addition of methionine and a hydroxy amino acid such as serine



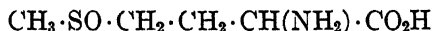
followed by cleavage, also resulting in cysteine. Binkley and du Vigneaud (7) have shown by the tissue slice method that homocysteine



gives rise to cysteine in the presence of serine, but have not shown that homocysteine is derived from methionine. An attempt was made in the experiments reported here to follow the course of the transformation in the presence of liver slices or brei, by utilizing a series of tests for the various specific groups in the substrates involved.

EXPERIMENTAL

The disappearance of added methionine has been followed in the present investigation by three methods, *a* the Lavine procedure² (8), *b* the peroxide oxidation (9) of the sulfur of methionine to the sulfoxide



and *c* the McCarthy-Sullivan colorimetric determination (10). The reaction products were analyzed for disulfides and cysteine by the Shinohara and Sullivan techniques. The presence or absence of α -keto- γ -methiobutyric acid was demonstrated by the precipitation of the 2,4-dinitrophenylhydrazone, as described by Waelsch (2, 3).

Loss of Methionine. 0.8 mM. of methionine was incubated with liver and kidney slices in 40 ml. of Krebs' salt solution at pH 7.4 for five hours at 38°C. with O₂ bubbling through the reaction mixture. The liver was first perfused with normal saline and the slices washed two times with Krebs' salt solution. Aliquot parts removed before and after incubation showed, with each method employed, a decrease in methionine of about 15–20 per cent in the protein-free neutralized filtrates. A second tube in which the methionine was omitted was the control. The presence of α -keto- γ -methiobutyric acid was demonstrated by its precipitation as the 2,4-dinitrophenylhydrazone.

When brei was substituted for slices, added methionine disappeared to the extent of 35–40 per cent as indicated by the Lavine method (8).

² The amino group is essential for this determination.

However, the peroxide oxidation determination (9) showed little or no loss of added methionine.

Employing benzoyl methionine, prepared as described by Windus and Marvel (11) as substrate, it was found that the peroxide oxidation values were similar to the values obtained from methionine. The Lavine method could not be used with this substrate.

In all cases cysteine determinations were negative.

Results are given in Table I.

TABLE I

Disappearance of Methionine Added to Liver Slices or Brei and Incubated Aerobically for Five Hours in Krebs' Salt Solution at pH 7.4 and 38°C.

Substrate	Tissue	Concentration of added methionine in mM per ml.								
		Lavine determination			H ₂ O ₂ determination			McCarthy-Sullivan method		
		Before	After	Δ	Before	After	Δ	Before	After	Δ
				per cent			per cent			per cent
Methionine	Slices	0.0190	0.0157	-17.4	0.0195	0.0165	-15.5			
"	"	0.0194	0.0160	-17.5	0.0188	0.0161	-14.7			
"	"	0.0186	0.0150	-19.7	0.0187	0.0156	-16.8			
"	Brei	0.0205	0.0141	-31.2	0.0195	0.0207	+6.1			
"	Brei	0.0211	0.0129	-38.9	0.0213	0.0201	-5.6			
Benzoyl methionine	Slices				0.0204	0.0161	-20.7			
"	"				0.0182	0.0157	-13.7			
"	"				0.0195	0.0173	-11.5	0.0200	0.0168	-11.0
"	Brei				0.0194	0.0194	0.0	0.0191	0.0202	+5.8

Demonstration of Cysteine Formation: (a) From Methionine. Since it was probable that any cysteine produced from added methionine was undergoing further oxidation, sodium pertitanate was employed as an inhibitor. This reagent was prepared by a modification of the procedure of Bernheim and Bernheim (12). 200 mg. of $\text{Ti}_2(\text{SO}_4)_3$ were added to 100 ml. of 0.05 M phosphate buffer at pH 7.4. A precipitate of titanous phosphate formed, 10 drops of 30 per cent superoxol were added until the mixture was definitely yellow and it was allowed to stand for 24 hours at room temperature. The solution was filtered, the filtrate boiled

vigorously to destroy excess H_2O_2 and kept at room temperature several days before use. The experiments described above were repeated, but with the addition of 2 ml. of sodium pertitanate. The Shinohara and Sullivan tests on the neutralized protein-free filtrates indicated that about two per cent of the added methionine had been converted to cysteine.

In another set of experiments, 1 mM. of methionine was dissolved in 20 ml. of Krebs' salt solution, and 2.00 ml. of the sodium pertitanate solution added, followed by the slices. Methionine was omitted from the control tube. After 5 hours of aerobic incubation the solution was decanted from the tissue, 2.00 ml. of 50 per cent trichloroacetic acid were added, and the flocculated protein was centrifuged out. The supernatant liquid was decanted into a 50 ml. centrifuge tube, and the pH adjusted to 4.5 using bromocresol green as external indicator. Fifteen drops of the cuprous chloride, prepared as by Rossouw and Wilken-Jordan (13) were added and it was allowed to stand for forty minutes before centrifuging. The supernatant liquid was decanted; 12 ml. of 0.1 N HCl and a pinch of NaCl were added to the precipitate, and H_2S was passed in until the copper was completely precipitated. This was filtered and CO_2 bubbled through the filtrate for 20 minutes. The Sullivan and Shinohara tests gave values corresponding to about 1 per cent of the added methionine, as recorded in Table II.

Added serine or neutralized sulfinic acid (as possible precursors of the non-sulfur portion of cysteine) failed to increase the yield of cysteine. Glycocyamine (employed as a potential methyl acceptor) also had no effect. Shifting the pH from 6.7 to 8.0 resulted in no measurable increase. α -keto- γ -methiobutyric acid, synthesized as described by Cahill and Rudolph (14) did not give rise to cysteine. Bicarbonate buffer yielded slightly higher values than did the phosphate buffer.

Under anaerobic conditions, with either nitrogen or nitrogen-carbon dioxide gas mixture, cysteine is released from the protein with or without added methionine. Experiments were inconclusive in attempting to decide if more cysteine resulted in the tubes containing the added methionine than in the control tubes.

When the tissues were heated to 85°C . for five minutes before incubation, the Sullivan determination was negative. However, the Shinohara, on the protein-free neutralized filtrate, indicated (if calculated as cysteine), a 1-2% yield of cysteine-even in the controls. However, after the cuprous chloride precipitation, only the tubes to which the

methionine had been added gave a strongly positive Shinohara test. The controls were very low.

(b) *From dl-homocystine.* 1 mE. of homocystine and 1 mM. of choline chloride, in 20 ml. of Krebs' salt mixture were incubated aerobically for five hours with tissue slices and two ml. of sodium pertitanate. Complete solution of the homocystine did not take place. As the yield of cysteine was much lower in this series of experiments than from methionine, the copper salt was taken up in only 7 ml. of HCl. The cysteine formed, as shown by the Sullivan reaction, amounted to 0.25% of the added homocystine. In controls in which the choline chloride was

TABLE II

Percentage of Cysteine Produced from 1 mM. of Added Methionine Incubated Aerobically with Liver Slices for 5 Hours in Krebs' Salt Solution at pH 7.4 and at 38°C.

Substrate	Tissue	Cysteine from added methionine	
		Shinohara	Sullivan
		<i>per cent</i>	<i>per cent</i>
Methionine.....	Liver Slices	1.56	1.34
Methionine.....	" "	1.80	0.72
Methionine.....	" "	1.56	1.12
Homocystine plus Choline . . .	" "		0.26
Homocystine plus Choline.....	" "		0.35

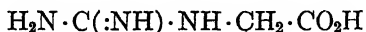
omitted, the homocystine gave rise to a very faint, doubtfully positive Sullivan test.

DISCUSSION

Although the foregoing experiments demonstrate that cysteine is produced in small amounts from methionine added to tissue slices, no clear pathway for this conversion (if it is a conversion) can be deduced, because of the small amount of cysteine formed, a yield that could not be increased. du Vigneaud (7) has shown a possible pathway for this conversion through homocystine and serine under anaerobic conditions. If we consider the formation of cysteine from methionine, under our conditions (aerobic), as a second pathway, then the inability of homocystine to act as a precursor of cysteine would eliminate it as an intermediate in the conversion. However, if a methyl donor is present, such

as choline chloride, homocystine gives rise to small amounts of cysteine. Attempts to determine the presence of methionine, as an intermediate, at this stage failed.

The yield of cysteine in each of these experiments was very low. Serine, employed as a cysteine precursor, failed to increase the yield. Cysteine sulfinic acid is easily converted to sulfate by tissue slices in good yields (9). If the sulfur portion of this compound is removed, theoretically the chain, $-\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$, remains. The possibility that this residue, or some modification of it, unites with methionine was considered, but the addition of neutralized cysteine sulfinic acid did not increase the yield of cysteine in our experiments. A possible accelerator, glycocyamine



also failed to produce more cysteine (15).

Another possibility to account for the appearance of cysteine in these experiments would be the freeing of cysteine from the tissue protein by methionine. This would explain our inability to increase the cysteine yield by the addition of possible precursors or accelerators.

It is of interest to note the similarity in the conditions required for sulfate production and cysteine formation. In both cases, slices (intact cells) are required for the reaction to take place. With brei, no sulfate is produced from methionine or other sulfur-containing amino acids. In the methionine-cysteine system brei does not change the sulfur titer of the methionine, and only the Lavine determination indicates a loss of methionine, through an oxidative deamination. The yields of sulfate and cysteine from methionine are also in the same order, about one to three per cent. Attempts by various investigators to increase the yield of sulfate from sulfur-containing amino acids have not as yet been successful. Evidence therefore points to a system which requires the intact cell for this possible conversion. Since no cysteine could be demonstrated when α -keto- γ -methiobutyric acid was employed as substrate it probably is not an intermediate in this scheme.

SUMMARY

Methionine incubated aerobically with liver brei is deaminated, but the sulfur remains intact. With slices, both deamination and a reduction in the sulfur titer occur. At the same time cysteine is produced. Homocystine gives rise to much smaller amounts of cysteine and then only if choline is present.

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Thiamin in Lake Waters and Aquatic Organisms

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INTRODUCTION

Harvey (1939) has indicated the presence and seasonal variation, in sea water, of at least two incompletely identified organic compounds necessary for the growth of the diatom, *Ditylum brightwellii*; like that organism, these substances are found in the water of the English Channel, mainly in winter. One factor, provisionally termed "A," could be replaced by cystin, thiamin, or by a crude biotin preparation, though the quantities of these substances required are far in excess of the amounts likely to be present naturally. In initiating a study of accessory organic compounds in lake waters, an examination of thiamin and biotin concentrations was therefore considered to be a convenient point of departure. Unfortunately pressure of other more urgent work, and the difficulties of transportation, have necessitated the curtailment of this program at an early stage, but a few results have been obtained that are of sufficient interest to merit publication.

TECHNIQUE

The determination of thiamin was made by Schopfer's method of bioassay with the fungus *Phycomyces blakesleeanus*, under the conditions described by Burkholder and McVeigh (1940). The growth of this organism in an otherwise adequate culture medium is dependent on the quantity of thiamin (vitamin B₁) present. With a given medium, within the correct pH range, and with standard conditions as to volume, temperature, etc., the weight of mycelial mat produced is remarkably constant. It is not a linear function of the thiamin present, and a calibration curve must be constructed when the quantity of thiamin to be estimated is in excess of 0.05 γ . For lower values, normally encountered in the present work, 1 mg. of dry mycelium may be taken as equivalent to 0.002 γ thiamin. The method does not distinguish between the thiamin molecule as such, and an equimolecular mixture of pyrimidine and thiazole. Slight errors may be introduced by the presence of amino-acids introduced with the sample, but under the conditions of the experiments to be described, they are not likely to be significant. The

determination of thiamin in organisms or in mud is performed merely by adding the sample, usually dry, to the culture flask, but in the case of lake waters it was found that preliminary concentration of the thiamin was necessary, the amount present being below the limits of sensitivity (about 0.0015 γ in 5 ml.) of the method. The concentration is performed by bringing the water to pH 3.8-4.0 with dilute H_2SO_4 , and evaporating 1 liter on a hot plate until 50-75 cc. of water remain. The pH is then adjusted to pH 4.5, and 5 cc. aliquots added to the culture flasks. Greater concentration would often be desirable, but it has seemed best to avoid as far as possible the massive precipitation of calcium sulphate and organic matter that may appear when the sample is boiled down further. An experiment in which thiamin was added to acidified lake water prior to concentration and compared with a second sample to which the same amount was added, but after concentration, indicates that no appreciable loss occurred during the boiling.

The most important ecological problem relating to thiamin in lake waters is that of the presence of the substance free in solution, where it might be available for species that, while otherwise autotrophic, are dependent on an external supply. A certain demonstration of the presence of dissolved thiamin has proved unexpectedly difficult. Blue-green algae tend to be less dense than water and are not adequately removed by the centrifuge, and even when these organisms were not present, no facilities for centrifuging large volumes of water were available. All organic filters, all apparatus of which rubber rings, etc., are an integral part, appear liable to contaminate the samples; in view of the low concentration of thiamin under study and the considerable reduction in volume necessary to detect it, contamination that would otherwise be negligible becomes serious. It has been necessary to discard several important series of determinations because evidence of contamination of distilled water blanks was obtained. The most satisfactory method of removal of the plankton so far employed is the simplest, namely filtration through no. 44 Whatman paper that has been boiled in sodium bicarbonate solution. It is essential that the Buchner funnel, glassware, etc., be kept as clean as possible. With the alkali-treated paper we have obtained filtrates of distilled water that, on concentrating, are apparently quite free of thiamin, but the treatment does not improve the texture of the paper and even with several filtrations the removal of plankton is certainly not complete. The few determinations of biotin were made by assay with *Saccharomyces cerevisiae* as described by Snell, Eakin, and Williams (1941) on two milliliters of unconcentrated water. In the single set of determinations that seem satisfactory, no difficulty was experienced in removing plankton by centrifuging the small volumes required.

Thiamin in Unfiltered Lake Waters

Preliminary studies on several lake waters were made in the summer of 1941, and a more detailed investigation of Linsley Pond, North Branford, in 1942. The localities, with the exception of Smith Brothers Pond, a eutrophic farm pond in the town of Southington, are described by Riley (1939), Hutchinson (1941), and by Deevey (1941). The results obtained are given in Table I.

Even in the short series of localities studied, it is clear that a rough correlation exists between the crop of plankton and the thiamin present. The first two lakes, in the Western Highlands of Connecticut, had light algal blooms. Linsley Pond, throughout the investigation, supported a large population of *Oscillatoria* (mainly *O. prolifica* Gom.) as well as other planktonic algae. The water of Smith Brothers Pond was a green soup of Myxophyceae, mainly *Polycystis aeruginosa* Kutz.

The 11 m. sample from Linsley Pond, obtained when thermal stratification had isolated the deeper water for several months, shows no tendency for thiamin to collect in the hypolimnion, as many inorganic nutrient substances accumulate.

TABLE I
Thiamin in Unfiltered Lake Waters

Lake	Date	Depth	Thiamin γ per liter
Bantam	15 July, 1941	0 m.	0.029
Lake Waramaug	15 July, 1941	0 m.	0.035
Linsley Pond	Range 1941-1942	0 m.	0.11-0.29
	Mean (six determinations)	0 m.	0.20
" "	1 September, 1941	0 m.	0.23
" "	1 September, 1941	11 m.	0.12
Smith Brothers Pond	10 July, 1941	0 m.	1.9

Thiamin in Filtered Water from Linsley Pond

As an example of the results obtained from replicate samples of filtered and unfiltered water, the determinations for surface water, collected on 1 Dec., 1941, may be considered, as the thiamin was also determined in the dried seston (plankton + detritus), collected on a membrane filter from this water.

	Replicate 1	Replicate 2	Mean
	γ per liter		
Unfiltered water	0.142	0.260	0.201
Distilled water passed through filter	0.0	0.0	0.0
Filtered water	0.078	0.076	0.077
Thiamin in seston (by difference)			0.124
Thiamin in 7.6 mg. seston (by direct determination)			0.114

In spite of the unusually wide variation in the results from the two unfiltered replicates, the seston thiamin estimated by difference is in good agreement with the value found directly.

The determinations set out in Table II, in general based on three cultures from each of two replicates, are regarded as satisfactory.

It appears that although the thiamin concentration of the filtered water varies from about 7 per cent to 39 per cent of the unfiltered water, the mean value of 24 per cent cannot possibly be explained entirely by algae leaking through the filter. Since the bacterial content of the free waters of lakes is normally negligible compared with the biomass of the autotrophic plankton, we may safely regard the greater part of the thiamin in the filtered water as either in solution or adsorbed on colloidal particles. At least some of this thiamin presumably is available to

TABLE II

Thiamin Content of Filtered and Unfiltered Water and of Seston. Linsley Pond. 1941-1942

	Unfiltered water	Filtered water	Seston by difference	
	γ per liter	γ per liter	γ per liter	$\gamma/\text{mg.}$
1 Dec. 1941 . .	0.201	0.077	0.124	0.016
2 Feb. 1942 . . .	0.112	0.008	0.104	0.024
29 April 1942	0.285	0.065	0.225	0.049
25 May 1942 . .	0.216	0.043	0.173	0.049
mean	0.203	0.048	0.155	0.035

unicellular aquatic organisms and though the amounts involved are small they are doubtless significant for the following reasons.

There is abundant observational evidence that phytoplankton populations can develop in lakes in which the concentration of ionic phosphate phosphorus is maintained at a level of 1-3 γ per liter; in Linsley Pond the concentration is seldom greater. Even if but half the thiamin in the filtrate were available, the ratio of ionic phosphate phosphorus to thiamin would be of the order of 100:1 in Linsley water. The mean phosphorus content of the dry seston (= plankton + detritus) of Linsley Pond is 0.35 per cent, in the detritus-free algal cells it is probably higher (Hutchinson, 1941). The mean thiamin content of the seston is, from Table II, 0.0035 per cent. It would appear probable, therefore, that any organism taking up thiamin and phosphate equally easily from the water is supplied with an adequate amount, or even an excess, of the

former substance. In lakes such as Waramaug and Bantam, in which only 0.03–0.04 γ thiamin per liter are found, the available supply in the seston-free water would clearly be lower. Extremely oligotrophic lakes would undoubtedly contain less. These results suggest that there is a *prima facie* case for investigating the accessory nutrient requirements of planktonic diatoms, particularly species such as *Fragilaria crotonensis*, *Synedra ulna* and *Asterionella formosa*, that have become abundant in Linsley Pond, as Dr. Ruth Patrick will demonstrate in a forthcoming paper, only since European settlement and intensive cultivation of the basin. There is, moreover, in the data of Table II, a distinct indication of seasonal variation of the thiamin content of both water and seston, which variation may ultimately prove interesting.

Thiamin in Aquatic Organisms

The thiamin content of certain whole invertebrates has been studied by Woods, Taylor, Hofer, Johnson, Lane, and McMahan (1942). They conclude that the smaller invertebrates, such as *Drosophila* larvae and the protozoan *Tetrahymena geleii*, contain more thiamin than do the larger invertebrates such as the earthworm *Lumbricus terrestris* or the oyster (*Mytilus*, sic) while most vertebrates contain even less than the larger invertebrates. At first sight the results obtained in the present study, and set out in Table III, seem in accord with this view, all our animals being small and our figures high. No great emphasis should be placed on the determination for *Synchaeta*, as but 0.3 mg. of the rotifer could be assembled, by phototaxis, for the analysis. The mean of all the zooplankton determinations, namely 38 γ per gram dry matter, is practically identical with the 37 γ per gram dry, recorded by Woods, *et al.* for *Tetrahymena*, and seven times the amount found in the whole rat. There must, however, be considerable variation of size within a given class of thiamin contents. *Tetrahymena* is much smaller than the average zooplankton. The cockroach, *Periplaneta americana*, contains 4.4 γ per gram wet or 16.3 γ per gram dry, which figures are in excess of those for *Mesocyclops*, certainly not more than one ten-thousandth of the mass of the cockroach. Possibly the relation merely implies that the lower metazoa, mostly small, are able to store thiamin in their tissues more effectively than can homiothermal vertebrates.

It is interesting to note that the thiamin contents per unit of dry matter in the two benthic dipterous larvae are almost identical with those recorded for larvae of *Drosophila virilis* (24.6, 23.1 γ per gram) by Woods, *et al.*

other easily digested parts of the mud. The quantity of thiamin in the only predator studied, namely the fairy-larva *Chaoborus*, is as great as or greater than in its prey (copepods and perhaps young *Chironomus*). All these benthic species are obviously good sources of thiamin for bottom-feeding fish.

Biotin in Linsley Pond water

A few determinations were made in the summer of 1942. Unfortunately the parallel thiamin determinations were unsatisfactory. The blank determinations were done on distilled water washed through the centrifuge tubes and other glassware employed. The best series relate to Linsley Pond surface water on 15 July, 1942.

	Replicate 1	Replicate 2	Mean
	<i>γ per liter</i>		
Blank.....	0	0	0
Uncentrifuged water.....	4.3×10^{-3}	3.5×10^{-3}	3.9×10^{-3}
Centrifuged water.....	2.5×10^{-3}	4.3×10^{-3}	3.4×10^{-3}

Removal of the algae from this water appears to have no significant effect on the biotin content, within the limits of error, which are considerable as the quantity present is little above the minimum detectible by the method. In this respect biotin appears to be distributed differently from thiamin, but much of the biotin of the plankton may not have been liberated in the uncentrifuged water.

The assays of prepared samples were carried out in the Osborn Botanical Laboratory by Dr. Paul R. Burkholder to whom my very best thanks are due; grateful acknowledgment is also made to Dr. Francis Drouet for determining certain blue-green algae, and to Dr. W. T. Edmondson and Mr. T. S. Austin for help in the field.

SUMMARY

The thiamin content of unfiltered lake waters lay between 0.03 γ and 1.2 γ per liter. In Linsley Pond from 7 per cent to 39 per cent cannot be removed by filtration, but reasons are given for supposing that even if only half the thiamin apparently in solution were available to planktonic algae, the concentration might be biologically significant. Seasonal variations in both the thiamin per unit dry weight of seston, and the thiamin in solution clearly occur. No accumulation was found in the hypolimnion at the end of stagnation. Aquatic invertebrates are rich in thiamin. The biotin content of Linsley Pond water in the summer appears to be about 3×10^{-3} γ per liter.

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Studies on the Mercapturic Acid Synthesis in Animals

XIII. The Relationship between Growth Inhibition in Rats by Bromobenzene and Mercapturic Acid Synthesis

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INTRODUCTION

White and Jackson (1) observed growth inhibition of rats upon administration of bromobenzene with a low casein diet. They attributed the inhibition of growth to the loss of cysteine by the rat for detoxication purposes, more specifically, for the synthesis of *p*-bromophenylmercapturic acid from the administered bromobenzene.

These observations of White and Jackson (1) have been extended to other substances, some of which, such as naphthalene (2), are known to yield the corresponding mercapturic acids in the rat. Other substances which also inhibited the growth of rats on a low casein diet have not been shown as yet to conjugate with cysteine *in vivo* (3).

Baernstein and Grand (4) working with inorganic derivatives of lead employing the experimental conditions of White and Jackson (1), expressed the opinion that "the reduced food intakes during the period when toxic substances were fed account satisfactorily for the reduced rate of growth observed in most cases." We suggested previously in connection with the metabolism of naphthalene in the rat (2) that "a study of the quantitative relationship between the intake of cystine and methionine and the output of cysteine as the mercapturic acid, correlated with the responses in growth, would, perhaps, provide information of value in ascertaining whether or not the withdrawal of cysteine

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as mercapturic acid is the sole factor responsible for the cessation of growth of rats on low casein diet ingesting toxic substances." The present work was therefore undertaken.

EXPERIMENTAL

Six groups of six male rats, of approximately the same age (30 to 35 days) and weight (60 to 70 g.) from several litters were used. The animals were kept in individual metabolism cages. The composition of the diets used is shown in Table I.

TABLE I
Composition of Diets

Diet	C-30 <i>per cent</i>	C-20 <i>per cent</i>	C-10 <i>per cent</i>	C-6† <i>per cent</i>
Casein*	30	20	10	6
Sucrose	15	15	15	15
Corn starch	26	36	46	60
Yeast powder	5	5	5	
Salts†	4	4	4	4
Crisco	15	15	15	15
Cod liver oil	5	5	5	

* Commercial casein containing 0.7 per cent of sulfur as cystine and methionine by analysis.

† Osborne and Mendel salt mixture Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.* **37**, 572 (1919).

‡ In addition to the diet, each rat received daily 400 mg. of yeast powder and 100 mg. of cod liver oil.

The plan of the experiments was as follows: One group was fed Diet (C)-30, and the second group was fed the same diet supplemented with one per cent of bromobenzene. The urine was collected from the rats ingesting bromobenzene every three days, for a period of forty days. The rats in both groups were weighed twice a week, and a record of food consumption was kept for the entire forty day period.

The urine was analyzed for *p*-bromophenylmercapturic acid by Stekol's method (5). The amount of cysteine lost by the rats during the ingestion of bromobenzene was then calculated and expressed in terms of casein sulfur. The amount of casein sulfur which was *actually available* to the rat for growth purposes while on the bromobenzene diet was determined by subtracting the sulfur lost as the mercapturic acid from that ingested. Another two groups of rats were then given a diet the casein concentration of which was adjusted to that calculated as avail-

able for growth in the previous group of rats which were fed bromobenzene. One of the groups received one per cent bromobenzene in the diet. The urine was collected and analyzed as before, the rate of growth and the food intakes of both groups of rats were also recorded for a forty day period.

Similar calculation of the loss of cysteine as the mercapturic acid was made for the second series of rats as for the first one, and a third diet was prepared the concentration of casein in which was equal to that calculated as *available for growth* of rats in the second series.

TABLE II

*Rate of Growth of Rats and Mercapturic Acid Excretion on Various Diets with or Without Bromobenzene**

Diet	Gain per day	Total		Sulfur		
		Food consumed	Sulfur ingested	Excreted as Mercapturic Acid	Available for Growth	As Casein Available in Diet
	<i>g.</i>	<i>g.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
C-30	3.6	366	768	—	768	30
C-30-Bromobenzene	3.7	348	741	261	480	19.7
C-20	3.2	317	444	—	444	20
C-20-Bromobenzene	0.9	333	462	219	243	10.4
C-10	0.9	263	184	—	184	10
C-10-Bromobenzene	-0.3	251	178	90	88	5

* Each group of 6 male rats was maintained on the diets described in Table I for 40 days. The data are average values per rat. Bromobenzene was fed in one per cent concentration of the diet.

The third group of rats was treated in the same way as was described for the first two. In this manner the rate of growth of rats was correlated with the intake of food, the concentration of available sulfur in the diet, and the sulfur lost as the mercapturic acid during the ingestion of bromobenzene. The results are presented in Table II. Bromobenzene was redistilled before use and the *l*-cystine was of known analytical purity.

DISCUSSION

The data in Table II show that the rats grew well on the 30 per cent casein diet, and the ingestion of bromobenzene did not inhibit the growth

in spite of the loss of cysteine as the mercapturic acid to the extent of making the amount of sulfur available to the rat for growth purposes equivalent to that present in a 20 per cent casein diet. On the 20 per cent casein diet bromobenzene inhibited the growth of rats markedly. The loss of cysteine as the mercapturic acid reduced the amount of available sulfur to that present in a 10 per cent casein diet. On the 10 per cent casein diet the inhibition of growth of rats by bromobenzene was complete. The amount of available sulfur on this diet was reduced by the ingestion of bromobenzene to that present in a 5 per cent casein diet. Comparison of the rates of growth of rats on the 30, 20, and 10 per cent casein diets with or without bromobenzene reveals the striking relationship between the rate of growth and the amount of sulfur, fed as casein, which was available to the rat. The loss of cysteine as the mercapturic acid accounted for the reduction in growth on diets with 20 and 10 per cent casein. The 20 per cent casein diet supplemented with bromobenzene induced practically the same rate of growth as the diet with 10 per cent casein unsupplemented with bromobenzene. The amount of sulfur lost to the rat made the 20 per cent casein diet with bromobenzene equivalent to the 10 per cent casein diet. The 30 per cent casein diet with bromobenzene was equivalent to the 20 per cent casein diet without bromobenzene, and the latter diet was quite satisfactory for good but not necessarily optimal growth. Hence no reduction in the rate of growth was observed on the 30 per cent casein diet with bromobenzene.

It is apparent from these data that the amount of cysteine lost as the mercapturic acid during the ingestion of bromobenzene determined the rate of growth of the rats. It can be concluded that, under proper dietary conditions, bromobenzene will inhibit the growth. The extent of the inhibition will be a function of dietary make up, particularly of the dietary sulfur-containing amino acids. The rate of growth under such conditions is determined by the ability of the rat to remove bromobenzene as the mercapturic acid, and by the concentration of the sulfur-containing amino acids in the diet only in so far as they enable the animal to repair the tissue injury during the process of detoxication.

In Table III are presented data on the extent of the synthesis of mercapturic acid in adult rats ingesting constant amount of food with bromobenzene for a period of 72 days. Diet C-6 was chosen because it was similar to that used by White and Jackson (1), and adult male rats were employed in order to ensure constancy in the consumption of food. The

rats excreted approximately constant amounts of the mercapturic acid up to 24th day. Thereafter the excretion of the acid decreased, and on the 30th day the amount of the acid excreted was about 50 per cent of that excreted on the preceding days. The rats appeared irritable and sensitive to handling. The animals lost about 35 per cent in body weight in 30 days. On incorporation of cystine into the diet which contained bromobenzene, and keeping the food intake the same as before,

TABLE III

*The Extent of the Synthesis of p-Bromophenylmercapturic Acid in the Rat During a Prolonged Daily Administration of 70 mg. of Bromobenzene With 7 g. of Diet C-6 With or Without l-Cystine**

Days on Diet	Mercapturic Acid Excreted in 3 Days	Days on Diet	Mercapturic Acid Excreted in 3 Days	Days on Diet	Mercapturic Acid Excreted in 3 Days
	mg.		mg.		mg.
3	87.3	27	62.4	51†	184.2
6	80.7	30	51.5	54†	171.6
9	98.9	33†	101.2	57†	175.6
12	97.2	36†	188.8	60†	184.2
15	94.6	39†	193.9	63†	168.7
18	105.8	42†	194.5	66†	157.3
21	103.1	45†	221.4	69†	157.9
24	76.7	48†	194.5	72†	169.3

* The initial weight of the rat was 275 g.; on the 30th day, 182 g.; on the 72nd day, 243 g.

† 0.5 per cent of l-cystine was incorporated into Diet C-6 containing one per cent of bromobenzene.

The data shown in this table are representative of those obtained on 6 animals.

the rats practically regained the weight lost and at the same time excreted for the next 42 days almost twice as much mercapturic acid per day as that excreted while on the same diet without cystine.

The data show conclusively that the loss in weight by the rats on the unsupplemented diet was due to the tissue catabolism induced by the demand for cysteine for mercapturic acid formation, and to the inability of the rat to meet this demand satisfactorily through adequate repair of the catabolized tissue at the expense of the diet. The incorporation of cystine into the inadequate diet improved the nutritive value of the diet and made thereby the needed material available to the rat for tissue

repair, and consequently, for a more efficient synthesis of the mercapturic acid from the ingested bromobenzene. Since the food intake throughout the 72 day period was kept constant, there appears little doubt that the efficiency in the detoxication of bromobenzene and the nutritive value of the food consumed were the paramount factors which determined the nutritional state of the animal.²

The data presented here offer experimental proof for the assumption that the inhibition of growth of rats by bromobenzene is due to the loss of cysteine as the mercapturic acid. Whether a similar conclusion is applicable to substances other than bromobenzene which were shown to inhibit the growth of rats under certain dietary conditions (3) remains, however, to be demonstrated.

SUMMARY

1. The rate of growth of rats maintained on diets of various casein content with or without bromobenzene was correlated with the cysteine excreted as the mercapturic acid and the organic sulfur of the diet which was available to the rat for growth purposes.

2. The rate of growth of rats was found to be a function of the available organic sulfur in the diet and of the extent of the detoxication of bromobenzene to yield *p*-bromophenylmercapturic acid. The assumption of White and Jackson (1) that bromobenzene inhibits growth of rats because of cystine deficiency created by the excretion of mercapturic acid in the urine is substantiated by experimental evidence.

A portion of the studies referred to herein were conducted, under the auspices of the International Health Division of the Rockefeller Foundation of New York City, by the Nutrition Units of the Departments of Biochemistry and Medicine of Vanderbilt University School of Medicine, Nashville.

² Upon quantitative consideration of data in Table III it will be noted that although 27.9 mg. of sulfur as cystine were supplied, only a range of 10 to 22 mg. of sulfur as mercapturic acid appeared in the urine in 3 days, whereas the 210 mg. of bromobenzene fed in 3 days called for an excretion of approximately 42 mg. sulfur as mercapturic acid. Obviously, bromobenzene fed was not excreted as mercapturic acid exclusively, as has been demonstrated earlier on numerous occasions. For the relationship between the synthesis of *p*-bromophenol and *p*-bromophenylmercapturic acid from bromobenzene the reader is referred to a recent review on detoxication mechanisms (6).

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Riboflavin and Pyridoxin

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INTRODUCTION

Previous studies (1, 2) have shown that twice as much thiamine and about 7 times as much choline (per gram of food) are needed for optimal growth response in tropical moist heat as compared to temperate coolness. No difference was found in the pantothenic acid requirement. Tests have now been completed on riboflavin and pyridoxin, revealing approximately equal needs for optimal growth in heat and cold. Proper adjustment of dietary vitamins now allows fully as rapid growth and development at 90°F. as at 68°F., although only $\frac{2}{3}$ as much food is consumed in the heat.

The purpose of these studies has been the analysis and remedy of the metabolic and growth depression engendered by tropical heat, and that aim now seems to have been accomplished. Animals grow with the same lusty vigor—are just as plump and rotund—in the heat as in the cold, provided they be given the extra supply of thiamine and choline.

EXPERIMENTAL

Weanling white rats (males) were placed in 2 air-conditioned chambers, one of which was kept at 68°F. and the other at 90–91°F. and 70% relative humidity. Weighings of the individual rats and their food consumption were made weekly. Basal diet used for the pyridoxin tests had the following composition:

Sucrose.....	76 g./100 g.	of diet
Casein (SMA, vitamin-free).....	18 " / "	" "
Corn oil.....	2 " / "	" "
Salts*.....	4 " / "	" "
Halliver oil.....	1.2 cc./kg.	" "
Thiamine chloride, cold room.....	1 mg. / "	" "
hot room.....	2 " / "	" "
Riboflavin.....	3 " / "	" "
Calcium pantothenate.....	.6 " / "	" "
Nicotinic acid.....	.25 " / "	" "
Inositol.....	1 g. / "	" "
p-Aminobenzoic acid.....	.03 " / "	" "
Choline chloride, cold room.....	.075 " / "	" "

The rats for each room were divided into groups of 4 and given access to unlimited quantities of the basal diet to which was added the following

TABLE I
Effect of Pyridoxin on Food Consumption and Growth in Heat and Cold

Weeks on diets	No. B6		0.5 mg./kg.		1 mg./kg.		2 mg./kg.		4 mg./kg.		6 mg./kg.	
	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten
Cold room												
First.....	16	51	23	56	23	60	25	61	26	62		
Second.....	12	65	17	71	19	78	22	90	23	90		
Third.....	7	53	15	69	19	78	23	100	31	103		
Fourth.....	6	48	9	62	25	92	22	103	28	101		
Fifth.....	5	51	7	62	20	95	35	115	26	109		
Sixth.....	-2	47	13	78	19	63	13	92	27	146		
Total for last 4 weeks....	16	199	44	271	83	328	93	410	112	459		
Body weight at end of 7 wks.....	82		129		184		205		217			
Hot room												
First.....	19	47	23	49	21	48	31	54	21	53	27	52
Second.....	9	46	18	59	20	57	22	66	31	67	26	70
Third.....	6	34	14	54	15	52	16	66	19	67	19	68
Fourth.....	5	34	17	63	23	51	22	72	29	66	25	53
Fifth.....	9	39	27	70	28	76	30	78	24	73	30	81
Sixth.....	7	43	15	71	21	78	20	77	22	78	18	96
Total for last 4 weeks....	27	150	73	258	87	257	88	293	94	284	92	298
Body weight at end of 7 wks.....	102		167		193		196		202		207	
Ratio: $\frac{\text{Food eaten}}{\text{Weight gain}}$												
Cold room.....	12.4		6.2		4.0		4.4		4.1			
Hot room.....	5.5		3.5		3.0		3.3		3.0		3.2	

increasing amounts of pyridoxin: group 1, no pyridoxin; group 2, 0.5 mg./kg.; group 3, 1 mg./kg.; group 4, 2 mg./kg.; group 5, 4 mg./kg.;

and group 6, 6 mg./kg. Table I presents the group differences in growth rate and food consumption.

TABLE II
Effect of Riboflavin on Food Consumption and Growth in Heat and Cold

Weeks on diets	No B ₂		1 mg./kg.		2 mg./kg.		3 mg./kg.		4 mg./kg.	
	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten	Weight gain	Food eaten
Cold										
First.....	23	58	25	60	27	61	27	62	26	61
Second.....	4	57	23	79	22	84	29	90	26	90
Third.....	3	55	15	87	32	99	33	107	24	100
Fourth.....	2	51	15	84	24	95	25	101	26	97
Fifth.....	-1	51	15	91	19	97	27	107	17	102
Sixth.....	1	57	19	88	16	90	14	102	29	106
Total for last 4 weeks...	5	214	64	350	91	381	99	417	96	405
Body weight at end of 7 weeks.....	71		165		188		197		196	
Hot room										
First.....	19	47	23	50	27	57	24	56	31	55
Second.....	5	47	18	67	24	70	23	70	29	71
Third.....	5	37	16	63	24	74	24	73	22	72
Fourth.....	7	37	20	63	26	78	28	73	21	63
Fifth.....	0	37	18	68	24	80	29	85	37	88
Sixth.....	0	39	18	71	27	90	20	65	20	69
Total for last 4 weeks...	12	150	72	265	101	321	101	296	101	292
Body weight at end of 7 weeks.....	12		181		216		202		227	
Ratio: $\frac{\text{Food eaten}}{\text{Weight gain}}$										
Cold room.....	42.8		5.5		4.2		4.2		4.2	
Hot room.....	12.5		3.7		3.2		2.9		2.9	

From the data presented, it would seem that 2 mg. of pyridoxin per kg. of food gives almost maximal growth in both heat and cold, although

there did occur a slight additional increase at 4 and 6 mg./kg. No significant differences in growth rate were observed in analogous hot and cold room groups, except that growth was more sharply retarded by the pyridoxin deficiency in the cold. Optimal intake at both temperature levels would thus seem to be 2-4 mg./kg. of diet. These rat findings are at variance with those previously reported for chicks (2).

Table II gives the results obtained on a similar series of rats fed graded dietary concentrations of riboflavin (0 to 4 mg./kg.). Diet mixtures were the same as described above for the pyridoxin rats, except for the graded amounts of riboflavin and the insertion of pyridoxin (2 mg./kg. for the cold room rats and 4 mg./kg. in the hot room). From the results obtained it is evident that no significant difference in requirement exists at the two temperature levels. The slightly better growth of all groups in the heat was probably due to the fact that their diets contained 4 mg. of pyridoxin per kg. while those in the cold had only 2 mg.

Similar series of rats in heat and cold were fed graded dietary concentrations of inositol and *p*-aminobenzoic acid, but without noticeable effects upon growth within 6 weeks time. Preliminary test of nicotinic acid requirement for the cure of canine blacktongue has shown no difference in heat and cold.

CONCLUSIONS

Riboflavin and pyridoxin requirements for optimal growth of young rats seem to be the same in both heat and cold. Inositol, *p*-aminobenzoic acid, and nicotinic acid likewise have shown no difference. Of all the B fractions, only thiamine and choline exhibit heightened requirements in tropical heat.

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A Rapid and Accurate Method for the Distillation of Ammonia Application to the Determination of Nitrogen, Ammonia, and Urea in Biological Fluids

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INTRODUCTION

The present method involves the vacuum distillation of ammonia obtained from urine, urea, or Kjeldahl digestion and its determination by absorption in a standard acid or Nesslerization. It is based upon a modification and adaptation of the apparatus described by Van Slyke, MacFadyen, and Hamilton (1) for the determination of carbon dioxide liberated from free amino acids by ninhydrin. The advantages of the method are the extreme simplicity, reproducibility, and accuracy of the procedure when used for the determination of ammonia nitrogen as low as 0.01 mg. In addition the apparatus is easily constructed from materials readily available.

APPARATUS

The apparatus is pictured in Fig. 1. Two sizes will be described, the dimensions given having proven quite satisfactory for our type of work. It is to be understood that these dimensions may be varied to suit individual needs.

The larger apparatus is constructed from 1" pyrex tubing (discarded test tubes are quite satisfactory). The length of the long arm is 9", the short arm 5", and the connecting arm 5". A 2" length of $\frac{5}{8}$ " pyrex tubing is attached to the short arm for insertion of the dropping funnel. The distilling vessel is a regulation 100 ml. Kjeldahl flask. The receiving vessel is a narrow mouth 125 ml. Erlenmeyer flask with the flange removed. The dropping funnel is constructed from a two-way capillary

stopcock, the lower end being attached to a 12" length of capillary tubing and the upper end to a cup designed to hold 10–15 ml. of reagent. It is attached to the apparatus through a one-hole rubber stopper. The receiving and distilling vessels are attached by means of 2" lengths of

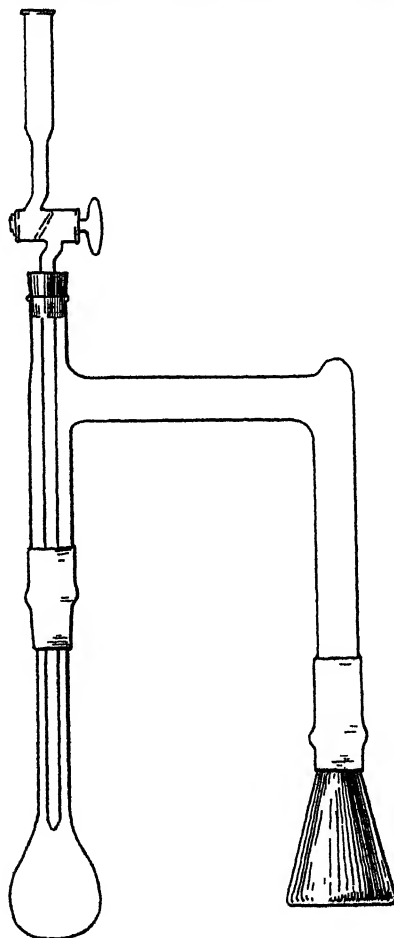


FIG. 1

heavy rubber tubing of $\frac{3}{4}$ to $\frac{7}{8}$ " internal diameter. It is important that the glass to glass ends be smooth and square.

The smaller apparatus is constructed in the same manner with slightly different proportions, using $\frac{1}{2}$ " pyrex tubing. The length of the long arm is $2\frac{1}{2}$ ", the short arm 2" and the connecting arm 5". The short arm

is continued upwards for 1" to provide an opening for the dropping funnel. The latter is the same as described above except that the stem is only 6-8" in length. For distillation a regulation 30 ml. Kjeldahl flask is used. A narrow mouth 50 ml. Erlenmeyer flask with the flange removed serves as receiving vessel, or it is replaced by a graduated Klett-Summerson colorimeter tube when the distillate is to be Nesslerized. Connections are made with 1½" lengths of ½" rubber tubing.

Hot and cold water baths are necessary for the distillation and should be so placed that the distilling and receiving vessels may be immersed simultaneously and held in place by a clamp. The temperature of the hot water bath should be 80-100°C. We find it convenient to run hot and cold tap water from adjacent faucets over the respective vessels when only an occasional determination is done.

A Klett-Summerson photoelectric colorimeter is used for the Nesslerized distillates.

REAGENTS

Sulfuric acid *N*/70, ⅓ *N* and 2.5 *N*.

Sodium hydroxide *N*/70, and concentrated (40 per cent).

Saturated potassium carbonate, about 90 per cent.

Phosphate buffer, 2.5 per cent potassium dihydrogen phosphate.

Urease, any good commercial preparation.

Indicators. Methyl red, 0.04 per cent in 95 per cent alcohol. Methylene blue, 0.02 per cent in water.

Caprylic alcohol.

Selenium digestion mixture. Gradually stir 250 ml. of concentrated sulfuric acid into an equal volume of water. (CAUTION!) Cool the mixture and saturate it with nitrogen-free K_2SO_4 using about 40 g. Add 1.0 ml. of selenium oxychloride and mix.

Nessler's reagent (Wicks (2)). Dissolve 51 g. of c.p. KI in 100-150 ml. of ammonia-free water. Heat this solution to 90-100° C. and add 16.2 g. of c.p. red mercuric oxide in small portions with stirring. Dilute this solution with an equal volume of water and allow to cool. Dilute 160 ml. of 50 per cent sodium hydroxide to about 500 ml., add the iodide solution, and dilute to one liter. One volume of solution is diluted with 5 volumes of water before use. The diluted solution keeps well but should be checked with standard $(NH_4)_2SO_4$ at weekly intervals.

Carbonate-oxalate reagent. Dissolve 100 g. of pure K_2CO_3 in 90 ml. of distilled water and boil for 5 minutes. Cool, add 10 ml. of saturated neutral potassium oxalate solution (30 per cent) and dilute to 140 ml.

Standard ammonium sulfate solution. Dissolve 0.472 g. of $(NH_4)_2SO_4$ in distilled water and dilute to 1000 ml. This contains 0.10 mg. of ammonia nitrogen per ml. Varying amounts and dilutions of this are used for preparing standards for use with the colorimeter.

TABLE I
Macroanalysis of Ammonium Sulfate Solutions

(NH ₄) ₂ SO ₄ 0.10 mg. N per ml. ml.	Found mg.	Ammonia N Per cent of theory
10.0	1.01	101.0
	1.00	100.0
	1.02	102.0
	1.00	100.0
15.0	1.51	100.7
	1.50	100.0
	1.50	100.0
20.0	1.98	99.0
	2.00	100.0
	2.00	100.0
25.0	2.50	100.0
	2.50	100.0
	2.51	100.2
	2.49	99.6
30.0	3.01	100.3
	3.00	100.0
	3.00	100.0
47.0	4.69	99.8
	4.71	100.2
	4.72	100.4
(NH ₄) ₂ SO ₄ 1.00 mg. N per ml. ml.		
10.0	10.06	100.6
	10.06	100.6
	10.08	100.8
20.0	20.21	101.0
	20.21	101.0
	20.27	101.3

GENERAL PROCEDURE

The Kjeldahl digestion flask containing the preformed ammonia is attached to the short arm of the apparatus, and the receiving flask containing the absorbing acid is attached to the longer arm. The dropping

funnel is attached to the apparatus and its open end attached to the water suction. The entire apparatus is evacuated to a pressure of 20-30 mm. of Hg (3 to 5 minutes), and the stopcock of the dropping funnel closed. The water pump is disconnected and the requisite amount of alkali placed in the dropping funnel. The alkali is cautiously run into the distilling flask, care being taken to exclude air. The vessels of the apparatus are immersed in the hot and cold water baths and the distillation allowed to proceed for three to five minutes. Observation of this upper limit of time is not essential in cases where no substances are present that may be converted to ammonia by prolonged heating with alkali.

When the distillation is complete air is allowed to enter the apparatus by opening the stopcock. The receiving flask is then disconnected and the ammonia determined by back-titration or Nesslerization.

RESULTS WITH AMMONIUM SULFATE SOLUTIONS

The results of a series of analyses with the macroapparatus are given in Table I. The ammonia was liberated in these determinations by the addition of 10 ml. of 40 per cent sodium hydroxide. The values given are unselected. Variations of ± 1 per cent are seldom encountered. The amount of *N*/70 sulfuric acid may be varied to suit the expected recovery of ammonia. 25.00 ml. was used in these determinations. Samples containing as much as 20 mg. of nitrogen have been satisfactorily distilled in this apparatus as shown in Table I. 0.1 *N* acid was placed in the absorbing vessel and 0.1 *N* base was used for back titration in these analyses.

Table II gives the results of a series of analyses with the microapparatus. 5.00 ml. of *N*/70 sulfuric acid was used to absorb the ammonia and back titration was carried out with *N*/70 sodium hydroxide from a 5.00 ml. microburette. 5 ml. of 40 per cent sodium hydroxide was used to liberate the ammonia. Variations of 5 per cent may be expected when the amount of nitrogen determined is less than 0.1 mg. Larger amounts should give an accuracy of 2 per cent.

Table III gives the results of a series of direct Nesslerizations of varying amounts of standard ammonium sulfate solution. In these analyses the required volume of solution was pipetted into the colorimeter tube, diluted to the 5 ml. mark with water, and 2.0 ml. of Nessler's reagent added. After standing for fifteen minutes the tubes were read in the colorimeter. Blanks on water read zero. The factor, *f*, is obtained by dividing the average reading of several determinations by the milligrams

TABLE II

Microanalysis of Ammonium Sulfate Solutions

(NH ₄) ₂ SO ₄ 0.05 mg. N per ml. ml.	Found mg.	Ammonia N Per cent of theory
1 0	0.048	96.0
	0.046	92.0
	0.052	104.0
2 0	0.098	98.0
	0.100	100.0
	0.100	100.0
	0.102	102.0
3 0	0.148	98.6
	0.154	102.6
	0.152	101.4
	0.150	100.0
4 0	0.198	99.0
	0.196	98.0
	0.200	100.0
	0.200	100.0
5.0	0.250	100.0
	0.248	99.2
	0.246	98.8
	0.250	100.0
6 0	0.296	98.7
	0.298	99.3
	0.302	100.7
	0.300	100.0
8 0	0.399	99.7
	0.398	99.5
	0.402	100.5

of ammonia nitrogen in the sample divided by 0.01. C (the concentration of ammonia nitrogen in 0.01 mg. units) can be calculated from the equation:

$$C = \frac{R}{f} \quad (1)$$

in which R is the colorimeter reading.

Table IV gives the results of a series of analyses in which the distillate was Nesslerized. In these determinations the microapparatus was used and ammonia was absorbed in 1 drop of 2.5 *N* sulfuric acid. The colorimeter tube served as receiving flask. The ammonia was liberated with 5 ml. of 40 per cent sodium hydroxide and distilled for not less than five minutes. After admitting air to the apparatus the distillate was diluted to 5.0 ml., 2.0 ml. of Nessler's reagent added, the tube stoppered, and the solutions mixed by inversion. Readings were made after fifteen minutes.

KJELDAHL DIGESTS

The macro and micro procedures have been applied to the determination of total nitrogen of blood, blood filtrates, spinal fluid and filtrates,

TABLE III

Direct Nesslerization of Standard Ammonium Sulfate Solution

(NH ₄) ₂ SO ₄ 0.02 mg per ml. ml.	<i>R</i> Colorimeter readings	<i>R_a</i> Average of readings	$F = \frac{R_a}{\text{mg. N}/0.01}$
1 0	63, 62, 63 63, 64, 63	63	31.5
2 0	126, 126, 125 127, 126, 126	126	31.5
4 0	250, 252, 251 253, 253, 252	252	31.5

feces, wood, blood fertilizer, urine, casein glues, purified blood proteins, ascitic fluid, and leather. Excellent results have been obtained with all these substances when the selenium digestion mixture described above was used. Undoubtedly other digestion mixtures would be satisfactory, but we have found this to be an excellent all-purpose reagent. The procedure for the determination of urine total nitrogen is given below as an example.

Urine Total Nitrogen

To a sample of urine containing 2-4 mg. of nitrogen in a 100 ml. Kjeldahl flask add 3 ml. of the selenium digestion mixture. Boil until the concentrated mixture has become colorless and continue the heating for 10 minutes longer. Cool the flask, add 10 ml. of water and attach to the apparatus. Attach the receiving flask, containing 25.00 ml. of *N*/70

sulfuric acid and three drops each of methyl red and methylene blue, to the apparatus. Evacuate at the pump, detach the suction, add 10 ml. of 40 per cent sodium hydroxide, and distill for 3-5 minutes. Admit air to the apparatus, detach the receiving flask, and titrate the excess sulfuric acid with *N*/70 sodium hydroxide. Typical results on aliquots of urine are given in Table V.

TABLE IV

Recovery of Ammonia Nitrogen from Known Solutions

(NH ₄) ₂ SO ₄ 0.01 mg. per ml. ml.	Colorimeter readings	Found mg.	Ammonia N Per cent of theory
1.0	31, 30, 30 29, 31, 31	0.0099	99
2.0	63, 62, 63 64, 63, 63	0.02	100
4.0	125, 126, 125 127, 127, 126	0.01	100
5.0	158, 157, 159 158, 158, 157	0.05	100
10.0	318, 313, 315 314, 315, 316	0.10	100

TABLE V

Total Nitrogen of Urine by Kjeldahl Digestion

Vacuum distillation g. per 100 ml.	Regular distillation g. per 100 ml.
1.051	1.030
1.051	1.035
1.050	1.051

Urine Ammonia

Excellent results have been obtained with this determination. The short time required to complete an analysis is the main advantage of the modification.

Determination of Urine Ammonia. Into a clean 100 ml. Kjeldahl flask measure exactly 5.0 ml. of urine, which must be acid to congo red. A few drops of 2.5 *N* sulfuric acid may be introduced as a routine procedure. The flask is connected to the apparatus and the remainder of the

determination carried out exactly as described for the analysis of Kjeldahl digests, except that 10 ml. of potassium carbonate reagent is used in place of the sodium hydroxide. Distillation must not proceed for more than five minutes to prevent the formation of ammonia from other nitrogenous substances. Table VI shows the agreement obtained by this method and compared to the aeration procedure. Table VII shows the importance of limiting the distillation time to five minutes.

Urea Nitrogen

Recovery from Urea Solutions. About 50 g. of Mallinkrodt A.R. urea was recrystallized from 200 ml. of hot alcohol. The air dried crystals were further dried in a vacuum desiccator over concentrated sulfuric acid for 24 hours. Kjeldahl analysis with vacuum distillation gave 46.9

TABLE VI
Determination of Urine Ammonia

Vacuum distillation mg. per 100 ml.	Aeration mg. per 100 ml.
36.2	37.6
37.4	36.8
37.6	
37.3	
61.2	63.4
63.3	64.2
63.6	65.5

per cent nitrogen, theoretical 46.6. 0.2140 g. of this urea was made up to 1000 ml. (1.0 mg. N in 10 ml.). 25.00 ml. samples were used for analysis. Results are given in Table IX. The procedure was the same as described for blood urea.

Blood Urea Nitrogen, Macro. Into a clean 100 ml. Kjeldahl flask place 4.0 ml. of whole blood, 1.5 ml. of phosphate buffer, and a pinch (10–20 mg.) of urease powder. Incubate at 50–55°C. for thirty minutes. Place 25.00 ml. of N/70 sulfuric acid and three drops each of methyl red and methylene blue in the receiving flask. Attach the flasks to the apparatus and evacuate at the pump. Disconnect the apparatus from the suction and place 6 ml. of carbonate-oxalate reagent in the funnel. Allow this to enter the distilling flask and follow with about 0.5 ml. of capryl alcohol, care being taken to close the stopcock before air is admitted to the system. Distill for 3–5 minutes and determine the ammonia liberated by back titration with N/70 sodium hydroxide.

Blood Urea Nitrogen, Micro. This method may be used when only 0.1 to 1.0 ml. of blood is available. The accuracy depends almost entirely on the accuracy of measurement and dilution of the sample. Several determinations may be done on the larger sample. The procedure is based on the action of urease on diluted whole blood with subsequent deproteinization as described by Gentzkow (4). The ammonia is then distilled from the alkalized filtrate and determined by Nesslerization. Folin and Svedberg (3) describe a method for the direct distillation of hydrolyzed filtrates to which the urease has been added after deproteinization, a technique that may give rise to excessive foaming.

TABLE VII

Effect of Prolonged Distillation on Recovery of Urinary Ammonia

Time in minutes	Ammonia N mg. per 100 ml.
2	34.8, 34.6
4	34.8, 35.2
6	50.6, 36.8

TABLE VIII

Determination of Urea Nitrogen

Solution containing 0.10 mg. urea N per ml. ml.	Found mg.	Urea nitrogen Per cent of theory
25.00	2.51	100.4
	2.50	100.0
	2.51	100.4
	2.51	100.4

One volume of blood (0.1 to 1.0 ml.) is mixed with seven volumes of water in a 15 ml. centrifuge tube and 5–10 mg. of urease powder are added with shaking. The tube is stoppered and incubated for thirty minutes at 50–55°C. in a water bath. One volume of 10 per cent sodium tungstate solution is next added and mixed, followed by one volume of $\frac{2}{3}$ N sulfuric acid. The tube is stoppered, shaken well, allowed to stand for 10 minutes in water at room temperature, and then centrifuged for 30 minutes. 0.5–2.0 ml. of the supernatant fluid is transferred to a 30 ml. Kjeldahl flask which is then connected to the apparatus. For the Nesslerization procedure it is best to choose a volume of filtrate containing 0.01 to 0.10 mg. of nitrogen. For the titration procedure the sample should contain 0.10 to 0.50 mg. of nitrogen.

For Nesslerization the Kjeldahl flask is connected to the distilling side of the apparatus and a Klett-Summerson photoelectric colorimeter tube graduated at 5.0 ml. and containing one drop of 2.5 *N* sulfuric acid is attached to the receiving side. The dropping funnel is attached and

TABLE LX
Blood Urea Nitrogen, mg. per 100 ml.

Sample No.	Aeration	Macro- titration	Micro- titration	Micro- Nessleri- zation	Nitrogen determined mg.
1	22.3	25.4			1.0
	25.0	25.1			
		24.5			
2	17.7	20.6			0.82
	17.5	20.6			
		21.2			
3		7.0	6.8		0.07
		6.9	6.9		
			6.8		
4		20.5	20.5		0.1
		20.4	20.1		
			20.2		
5		29.0		28.4	0.029
		29.3		29.1	
				28.6	
6		63.4		62.2	0.068
		63.0		63.0	
				62.5	
7		9.5		8.9	0.018
		9.1		9.0	
				8.6	

the apparatus evacuated as described above. Two ml. of carbonate-oxalate reagent are added through the dropping funnel and the apparatus immersed in the water baths for from 3-5 minutes, during which time distillation is complete. The colorimeter tube is detached from the apparatus and its contents diluted to 5.0 ml. 2.0 ml. of Nessler's reagent are then added and the contents of the tube mixed by inverting.

The color is allowed to develop for 15 minutes, after which it is quite stable for at least two hours. If good checks on aliquot samples are desired it is essential to use calibrated pipettes and colorimeter tubes throughout.

The procedure for titration is exactly as described above except that a sample containing 0.10 to 0.50 mg. of nitrogen is used and the distillate collected in a 50 ml. Erlenmeyer flask containing 5.00 ml. of *N*/70 sulfuric acid. The ammonia is determined by back titration with *N*/70 sodium hydroxide from a 5.00 ml. burette. The indicator is the same as described above.

The results are summarized in Table IX.

SUMMARY

A simple and accurate method for the quantitative vacuum distillation of ammonia from Kjeldahl digests and other solutions is described.

The method has been applied to the determination of nitrogen, ammonia, and urea in biological fluids.

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The Glycogen Content of Various Parts of the Central Nervous System of Dogs and Cats at Different Ages

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INTRODUCTION

Though studies of brain glycogen have been made by several workers, Kerr (1), by the use of liquid air to insure glycostasis, was the first to obtain reliable values of brain glycogen averaging somewhat less than 0.1 % for the cerebral cortex of dogs, cats, and rabbits. In the present investigation of the glycogen content of the various parts of the central nervous system, sodium iodoacetate was used, because this substance is glycostatic for the whole neuraxis, whereas liquid air is active only on the surface of the brain which it comes in contact. Since the brain possesses characteristic patterns of morphological (2) and biochemical (3, 4, 5, 6, 7) development during growth, the glycogen content of different parts of the central nervous system were determined at three strategic growth periods.

METHODS

The animals used were newborn, 5-8 week, and adult cats and dogs. Except for one control experiment on a dog, the animals were not fasted. The parts of the central nervous system analyzed were the cortex, caudate nucleus, thalamus, corpora quadrigemina, medulla oblongata, and cord. When the amount of tissue was small, samples from several members of a litter were pooled; when it was large more than one determination was done on it. Effort was made to eliminate white matter from the cortex. In the case of the caudate nuclei, thalamus, colliculi, and medulla, each was taken in its entirety. From adults, sections of the cerebellum and cord; and from infants the entire cerebellum and cord were analyzed.

With slight modification the method of Kerr (1) was adopted. Sodium iodoacetate was used in place of liquid air to fix brain glycogen *in situ*. In most cases 200 mg. per kilogram were injected intraperitoneally. After the sodium iodoacetate had caused loss of consciousness, the brain was exposed. About 15-30 minutes after the injection,

TABLE I

Average Values of Glycogen Contents, mg. per 100 g., of Tissue of the Various Parts of the Central Nervous System at Three Different Ages

	Cortex	Caudate nucleus	Thalamus	Colliculi	Cerebellum	Medulla	Cord
New born cat	23 [12-32] (16)	—	48 [30-89] (5)	45 [33-68] (3)	107 [47-158] (7)	101 [72-164] (6)	137 [126-151] (3)
5 to 8 week old cats	45 [25-66] (15)	36 [32-43] (4)	30 [28-32] (3)	38 [37-36] (3)	97 [62-139] (7)	35 [22-18] (6)	47 [34-56] (6)
Adult cat	68 [42-120] (10)	46 [26-96] (9)	27 [19-39] (9)	28 [21-30] (6)	32 [10-53] (9)	31 [21-47] (8)	25 [10-53] (6)
Newborn dog	18 [14-23] (12)	34 [28-47] (6)	44 [20-88] (15)	60 [33-93] (12)	65 [44-98] (16)	122 [90-157] (12)	127 [75-200] (13)
5 to 8 week old dog	31 [24-45] (10)	39 [31-62] (10)	53 [35-74] (9)	56 [31-75] (9)	69 [50-91] (10)	58 [33-85] (9)	45 [20-63] (11)
Adult dog	73 [45-108] (7)	58 [44-71] (7)	62 [41-68] (6)	50 [37-60] (7)	35 [21-46] (6)	39 [21-47] (6)	29 [15-31] (6)

The numbers in brackets are the extreme upper and lower values. These values show the same general trend as the averages and similarly indicate which differences are significant.

Values in parentheses are the number of animals used.

the brain was removed, and the various regions were rapidly dissected into weighed test tubes containing the alcoholic potassium hydroxide solution. A few animals which had been narcotized with pentobarbital, were injected intravenously with 75 mg. per kilogram of sodium iodoacetate in a 2 per cent solution. This must be done slowly because rapid injection will stop the heart before sufficient sodium iodoacetate has been absorbed by the central nervous system for complete glycostasis. With slow injection respiration ceases before the heart stops.

The Hagedorn-Jensen method (8) was chosen in preference to that of Shaffer-Somogyi (9) since it permits determination of smaller aliquots of the glycogen hydrolyzate. In every instance yeast fermentation, according to the method of Raymond and Blanco (10) was carried out and the non-fermentable reducing substances determined. As a control on the method, samples of cerebral cat cortex were analyzed according

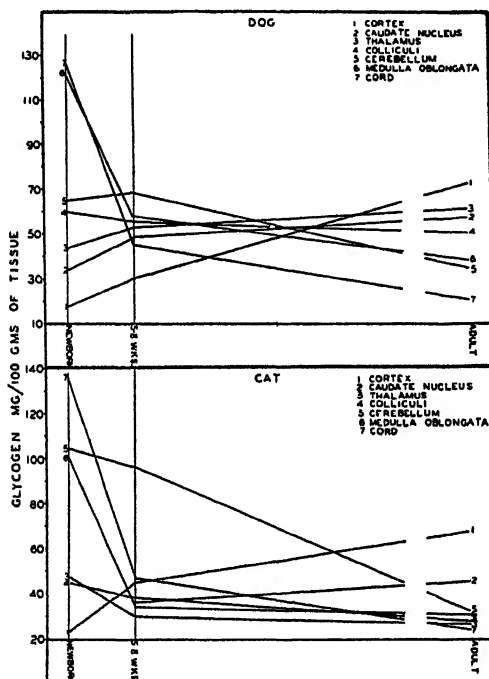


FIG. 1

Changing Relationships of the Glycogen Content in the Various Parts of the Neuraxis During Growth

to the technique of Kerr (1) using liquid air as the fixative and the Shaffer-Somogyi (9) method for the determination of reducing substances.

RESULTS

The average glycogen values for each part and the number of animals used are presented in Table I and Fig. 1. These results were analyzed according to Fisher's *t* test (11). The *p* value for each part of the brain

was calculated for the difference between the means of the newborn and the 5-8 week old, the newborn and the adult, and the 5-8 week old and adult animals. Only *p* values less than 0.05 were considered significant.

Cortex: The mean values for the dog and cat cortex exhibit a progressive increase in glycogen content from the newborn to the adult animal.

Caudate Nucleus: The caudate nucleus of the newborn dog contains significantly less glycogen than that of the 5-8 week or the adult dog. The caudate nucleus of the cat could not be compared in this manner because the combined nuclei of a litter of newborn kittens contained less glycogen than could be determined by this method.

Thalamus: Although the thalamus of the adult cat showed a significant decrease, the thalamus of the adult dog was significantly higher than that of the newborn.

Corpora Quadrigemina: In cats the only change in the glycogen content of the corpora quadrigemina was a decrease in the adult, and there were no significant changes in the dog.

Cerebellum: The cerebellar glycogen did not change from the newborn to the 5-8 week animals, but in both species there was a large significant fall from the 5-8 week old to the adult.

Medulla Oblongata: In both animals there is a large decrease in the glycogen content of the medulla from newborn to 5-8 week, and no further significant decrease with age.

Cord: Both the 5-8 week and the adult cats and dogs show a large reduction in the concentration of glycogen in the cord.

DISCUSSION

It has been established by Kerr (1) that the glycogen content of the adult brain is relatively stable. It is not increased by diet or decreased by convulsions or by any other conditions excepting those which produce anoxemia or hypoglycemia. This glycogen provides an additional source of carbohydrate for oxidation during hypoglycemia or for glycogenolysis during anoxemia. Cerebral glycogen acts as a buffer to protect the central nervous system in these states of emergency.

Our average values for cerebral cortex were 0.073% for dogs and 0.068% for cats as compared to the values of Kerr and coworkers of 0.098 for dogs, 0.086 for cats, and 0.082 for rabbits (1). Control experiments on cat cerebral cortex using liquid air and the Shaffer-Somogyi method (9), according to Kerr, yielded the same results we had pre-

vously obtained. Other observations made on the cortex of a fasted dog averaged 0.090%. Kerr similarly observed a slight increase with fasting. Other than the cortex, there are no previous results in the literature with which to compare the present observations of the glycogen content of the parts of the central nervous system.

In general the newest phyletic portions of the central nervous system show a progressive increase in glycogen content with growth while the oldest parts exhibit a decrease (Fig. 1). For the intermediate portions there is a slight rise in the thalamus and no change in the colliculi of the dog and a slight fall in both of these parts in the cat. It is of interest to note in this respect that Dr. Papez' histological findings show that the thalamus assumes the rôle of a higher center in the dog than in the cat. On the other hand the colliculi in the cat appear to retain the functions which in the dog have been taken over by the retino-geniculocortical structures (12).

In order to evaluate the significance of the differences in glycogen content of the various parts of the brain it must be recalled that the constituents of the brain change in concentration during growth. For example, the laying down of white matter or myelinization is completed only some time after birth (3, 6, 7). It is, therefore, probable that the fall in the glycogen content of the older parts of the brain represents a reduction in the proportion of gray matter rather than an actual decrease in the concentration of glycogen in the cells. On the other hand, the increase of glycogen in the newer parts of the brain is caused by an augmentation, which occurs despite a mixture of gray matter with white. The rise in the glycogen content of the cerebral cortex and caudate nucleus of both animals and the thalamus of the dog represents an actual gain while the decrease in the other portions may be only apparent due to the diminished proportions of gray matter.

The changes in the glycogen content of the various parts of the central nervous system are not isolated phenomena, but correlate with other observations. It has been previously found that the oxygen intake of the various parts of the brain of the dog is low at birth, with the phyletically newer portions lower than the others. A differential change then occurs, and the cerebral cortex and caudate nucleus which possessed the lowest metabolism at birth assume the highest rate as growth is completed (4). Similar results have been obtained in a study of the rat brain at varying stages during growth (5).

The changes in glycogen content also correlate with the changes in

choline esterase activity of the various parts of the fetal sheep and of the adult ox brain, which exhibit a similar order of development (13). Peculiarly enough both the choline esterase activity and the oxygen consumption of the adult caudate nucleus are higher than those of the cerebral cortex. Except for this difference in order between the caudate nucleus and the cerebral cortex, the changes in glycogen content, oxygen consumption, and choline esterase activity parallel each other during growth. These changes represent a portion of the biochemical background of cerebral function. The biochemical changes correlate with a neuro-muscular control which seems to advance in a rostral direction from the bulbo-spinal region toward the cerebral cortex (14). The late assumption of function by the cerebral cortex is observed not only in the changes of behavior but also in the development of the electrical potentials in the cat's cortex. The electroencephalogram of the kitten, which is practically devoid of activity at birth, exhibits a gradual increase in constancy, amplitude, and rhythmicity until growth ceases (15). This longitudinal development of the central nervous system is in accordance with the conception of its phyletic organization.

SUMMARY AND CONCLUSIONS

The glycogen content of various parts of the cat and dog brain were determined in newborn, 5-8 week, and adult members of each species. Changes of three different types were observed:

1. The glycogen content of the newest phyletic parts, the cerebral cortex and caudate nucleus of the cat and dog increased with age.
2. For the intermediate portions a moderate decrease was found in the thalamus and quadrigemina of the cat, while in the dog the thalamus showed a slight rise and the colliculi no change.
3. The percentage of glycogen of the oldest parts, the cerebellum, the medulla, and cord fell progressively both in the cat and in the dog.

These results are discussed in relation to the phyletic development of the central nervous system.

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An Electrolytic Method for Controlling Oxidation-Reduction Potential and Its Application in the Study of Anaerobiosis¹

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INTRODUCTION

The idea that the prevention of growth of obligate anaerobes by air may be determined by a certain level of oxidation-reduction potential, rather than by a specific effect of oxygen, logically followed the work of Clark and others (1) and was formulated by Quastel and Stephenson (2). The disparity between the limiting Eh levels for the growth of a variety of anaerobes as reported by several authors (3) is due, we feel, to the failure to maintain the Eh levels constant for a sufficient length of time. More convincing to us is the work of Knight and Fildes (4) on the limiting Eh level for the germination of spores of *Cl. tetani*, +0.110 volt at pH 7.2; and of Vennesland and Hanke (5) who reported that *Bacteroides vulgatus*, a non-spore-forming obligate anaerobe, does not grow at an Eh level more positive than 0.150 volt at pH 6.6; while at more negative potentials this organism grows regularly on a suitable medium. That the addition of reducing agents promotes the growth of obligate anaerobes (6) and the action of many enzymes (7), has frequently been pointed out. More recently the actions of papain (8), urease (9), and phosphatase (10) have been shown to depend upon certain Eh levels, which differ widely from each other. It appears, then, that oxidation-reduction potential, like hydrogen ion activity, is a determining factor in many enzyme actions, and it is to be expected that Eh, like pH, values and the factors which control them will be found to be of fundamental importance in many biological phenomena and metabolic reactions. Our investigations on anaerobes provide a striking illustration.

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Many observations on the Eh values of biological systems (11) have been reported, and in bacterial systems particularly the effect of growth on oxidation-reduction potential has been studied (12). In our work this point of view has been reversed, in that we are studying the effect of controlled Eh levels on growth. In order to keep the Eh value of an inoculated medium constant, it is necessary to counteract the reducing agents which are liberated by the growing system, and this can be accomplished by the controlled addition of oxidizing agents. Knight and Fildes (4) and Vennesland and Hanke (5) controlled the oxidation-reduction potential of their cultures by means of the proportion of oxygen to nitrogen which was continually passing through the medium.

In this study the potential is controlled by another method, namely direct current electrolysis. By placing in the medium a large platinum electrode which is attached to the positive pole of a direct current, while the negative pole is attached to a similar electrode in another solution which makes a sterile liquid junction to the medium, it is possible to liberate oxidizing agent (or remove reducing agent) at the electrode surface in the medium, and thus make the oxidation-reduction potential of the medium more positive. By attaching the electrode in the medium to the negative pole of the electrolyzing current, the medium may be made more negative. By choosing properly the current and duration as well as the direction of this electrolysis one can achieve a fine control over the oxidation-reduction potential of the solution. A feature of this method is that no extraneous or foreign substances need be added; the electrolysis achieves its control by the oxidation of such reducing agents or the reduction of such oxidizing agents as are inherently in the system. At the same time the gas tension may be chosen at will: pure N_2 , or air, or any mixture of these or other gases.

In this way it is possible to show that certain obligate anaerobes will grow in a continuous current of air, if the oxidation-reduction potential is kept sufficiently negative by direct current electrolysis. On the other hand, under completely anaerobic conditions, these obligate anaerobes will not grow on a nutrient medium if the Eh value is kept above 0.150 volt by positive electrolysis; when subsequently by electrolysis or otherwise the medium is made more negative than Eh 0.150 volt, growth consistently occurs. The limiting potentials for growth are the same whether controlled O_2 tension or controlled electrolysis is used to maintain the potential level. It is also shown here that the limiting potential for growth of *Cl. sporogenes* varies with pH, being a maximum of Eh

0.150 volt at pH 6.6, and somewhat lower, about Eh 0.135, at pH 6.0 and at 7.0. Finally the observations of Katz and Hanke (13) on the O_2 consumption by growing cultures of *Bacteroides vulgatus* have been confirmed and extended.

MATERIALS, APPARATUS, AND GENERAL PROCEDURE

Organisms and Media

The organisms used in this study were two obligate anaerobes of widely different types: *Bacteroides vulgatus*, non-spore-forming, and *Cl. sporogenes*, which is spore-forming. Cultures of the *Bacteroides vulgatus*, strain Marino, were kindly furnished by Dr. A. H. Eggerth of the Long Island College of Medicine. Cultures of the *Cl. sporogenes* were obtained from the Department of Bacteriology of the University of Chicago. Subcultures were made on Rosenow's brain-broth medium. For the potential studies glucose-nutrient broth was used; which consisted of 10 g. glucose, 10 g. sodium chloride, 5 g. Bacto-peptone, and 3 g. meat extract in 1 liter water adjusted to pH 7.0 before autoclaving. After autoclaving, the pH was about 6.7. Most rapid growth was obtained with inocula of 24-hour cultures of *Cl. sporogenes* and 48-hour cultures of *Bacteroides vulgatus*.

Electrode Vessel and Assembly of Apparatus

The electrode culture vessels (Fig. 1) consist of 4-oz. wide-mouth bottles, each of which contains 75 ml. of medium and is fitted with a rubber stopper in which holes are drilled to support the following: A—gas inlet tube, B—inoculating tube and gas outlet, C—two platinum wire electrodes, D—platinum foil electrode, E—burette with capillary tip, rubber connection, and pinch clamp, F—two salt bridges, and G—a short glass tube, 11 mm. outside diameter, which serves as the support for the glass electrode. The salt bridges consist of two L-shaped glass tubes, 5 mm. inside diameter, joined by rubber tubing with space for a screw clamp. Before autoclaving, the distal end of each salt bridge is immersed in a vial containing 3 per cent agar and 1 per cent NaCl, and, without removing air from the bridge, the clamp is closed. During the autoclaving the air is driven out, the bridges fill with fluid, agar on one side and medium on the other, so that after cooling a sterile liquid junction is made simply by opening the clamp. The vials with solid agar are removed and the free ends of the bridges are ready for use. The glass electrode, H, a small bulb sealed on the end of a glass tube 7 mm. outside diameter (see Vennesland and Hanke [5], p. 141, also Hanke [14], pp. 30-33) must be narrow enough to slip through the glass support, G. A short rubber sleeve fits over the glass electrode tube and makes an air-tight joint when inserted into the support, G. The glass electrode is sterilized by immersion for 5 minutes in 0.1 per cent $HgCl_2$, then it is rinsed with sterile distilled water, and

at once immersed in the previously autoclaved vessel containing the culture medium.

All experiments were carried out with the culture vessels inside of a special, electrically shielded incubator, consisting of a copper box, which serves as electrical shield, inside of a transit box electrically regulated at 38° C. By means of a copper tube the shield is made continuous with a vacuum tube potentiometer. We used a Hellige 7030 vacuum tube galvanometer, and a Leeds and Northrup 9655 potentiometer. From each platinum wire or glass electrode in the culture

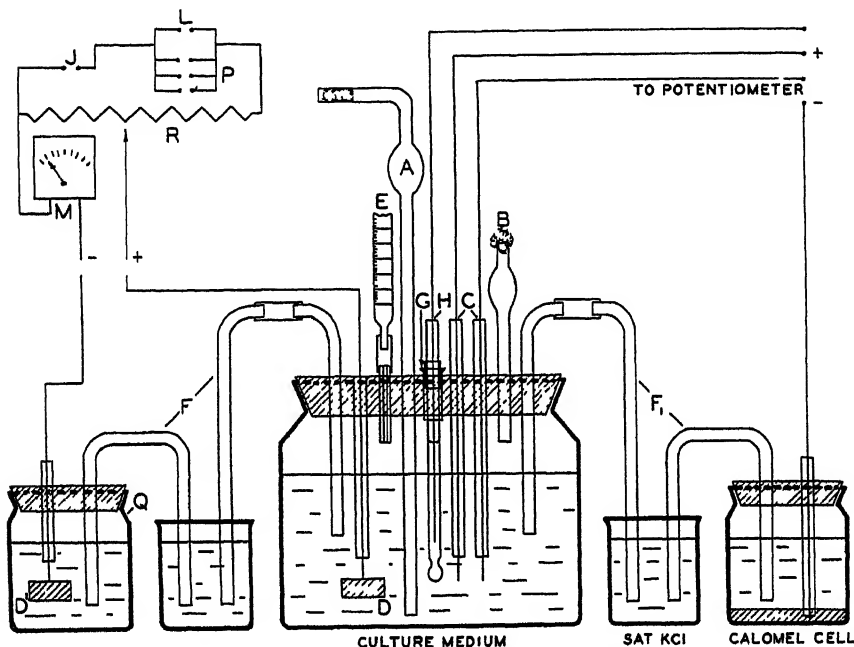


FIG. 1

vessel, by means of spring clips and insulated wires, connection is made to one of a series of permanent binding posts on the inner wall of the copper box, and each of these binding posts may be connected at will through a special selector switch, which can be manipulated from the outside of the box, to the binding posts of the vacuum tube galvanometer. In this way any one of the many electrodes may be instantly chosen for a reading without opening the box, or in any way manipulating the electrode vessels.

The electrolysis circuit, see Fig. 1, is entirely independent of the circuits for reading the potentials. Any one of a number of current sources and combination of resistances may be used. In our electrolysis circuit, the 110 volt direct current J, is led in parallel through a lamp, L, and a 3-heat 8-ampere hot plate, P, and then

in series through a 100-ohm rheostat, R. The current from the rheostat (one fixed and one movable contact) is used for the electrolysis, one pole of which is joined to the large foil electrode, D, in the culture medium, and the other through the milliammeter, M, to the large foil electrode, D', in the bottle, Q, containing saturated KCl. By altering the hot plate switch, and the movable contact of the rheostat, the voltage, and thus the current, in the electrolysis circuit may be varied at will. The maximum electrolysis current, with a single salt bridge of about 5 mm. bore, was about 20 milliamperes, with a voltage of about 30. More recently, with wider, shorter, and multiple bridges, this current has been increased to 120 milliamperes.

From the large foil electrode in the electrode vessel, connection is made through a spring clip to an insulated copper wire which protrudes and is held firmly about 1 cm. outside the transite box. A similar protruding wire makes connection to the large foil electrode in the saturated KCl which in turn is joined electrically through a salt bridge to the electrode vessel. Whenever the medium is to be electrolyzed, the electrolysis wires from the rheostat and the milliammeter ending in the spring clips with labeled polarity are connected to the two protruding wires leading to the large foil electrodes. While taking readings on the potentiometer these electrolyzing leads must be disconnected, because the induced currents disturb the sensitive vacuum tube measuring device.

Use of Dye to Poise the Culture Media

In order to improve the agreement between the readings given by two shiny platinum electrodes in the same culture medium, 2 ml. of 0.03 per cent toluylenic blue were added to each vessel (75 ml. of medium). For preparing this solution, about 3 mg. solid dye are put into a dry vial, and after plugging the vial with cotton, all is heated at 110°C. overnight. After cooling, 10 ml. sterile water are added, and the mixture is stirred for half a minute to cause solution. Heating the dry dye above 120° or autoclaving in solution causes decomposition. Without dye the readings given by two electrodes usually differ by 10 to 50 millivolts; after adding the dye the electrodes agree within two or three millivolts, when the reading is within 50 millivolts E_0 value of the dye (that is, between E_h of +0.100 to +0.200 volts at pII 6.5). In experiments where growth occurred, the poisoning effect of the dye gradually decreased and after six to ten hours entirely disappeared, due apparently to some chemical destruction of the dye. In such cases the color of the dye did not return even at potential levels where the dye should have been com-

pletely oxidized. Except for the effect of the oxidized form of the dye on the Eh, the presence of the dye was not observed to have any effect on the growth of these anaerobes.

Stirring and the Control of Gas Tension

In order to achieve adequate stirring and also to control the gas tension, gas mixtures were continually bubbled through the culture vessels at a rate of about two bubbles per second. The gas entered through the gas inlet tube, A, Fig. 1, and the constant surging of the gas bubbles effectively stirred the medium. For the anaerobic experiments this gas was purified nitrogen, obtained by passing tank nitrogen through glowing copper gauze in a quartz tube. Since the *Bacteroides vulgatus* required CO₂ for growth, the gases were passed through a saturated solution of sodium bicarbonate immediately before entering the culture vessels. While this CO₂ was not necessary for the *Cl. sporogenes*, it was routinely used in all experiments.

Comparison of Experimental and Control Vessels

Usually three vessels were studied simultaneously: two experimental, and one control. In the experimental vessels, the gas mixtures or electrolytic control of potential were chosen so as to maintain a certain level of potential. In the control vessel, a gas mixture of purified nitrogen and CO₂ was circulated without any electrolysis so as to provide optimum anaerobic conditions, while potential and pH were observed every half hour. In the controls, turbidity, our most definite evidence of growth, appeared in from eight to twelve hours, and a comparison of this time with the time required for turbidity development in the experimental vessels, was a criterion for the adequacy of the conditions in the experimental vessels.

Criteria for Growth

In general, growth was indicated by the successive appearance of three phenomena, increase in reducing power, development of turbidity, and fall in pH (see Vennesland and Hanke [5], p. 153). Increase in reducing power means either a marked increase in negativity of potential, or an increase in the amount of positive electrolysis or oxidizing agent that is needed to keep the potential constant. In the experimental vessels with electrolytic control of potential, the pH changes caused by electrolysis were often more rapid than those which could be

caused by growth, and so pH changes could not very well be used as a criterion for growth. Our most reliable index of growth was, after all, turbidity, and although this was a subjective observation, a comparison of the experimental vessels with the positive control on the one hand, and with a bottle of sterile medium on the other, always led to a very definite and semi-quantitative conclusion.

Tests for Contamination

At the end of each experiment, 0.2-ml. samples of each culture vessel were transferred to sterile tubes of nutrient broth saturated with air, and kept, first at room temperature for 24 hours, and then at 38° for 48 hours to test for aerobic contamination. Any tube showing development of turbidity was considered contaminated, and the results from such vessels where growth occurred were discarded. Contamination occurred in about 10 per cent of the vessels.

General Procedure for an Experiment

The successive steps of an experiment follow: The culture vessel containing 75 ml. of glucose nutrient broth and fitted with the rubber stopper containing the platinum electrodes, salt bridges, burette (but not the glass electrodes) is autoclaved for 20 minutes at 15 pounds pressure. The control vessel, not containing the large foil electrode or the burette, and having only one salt bridge, is simultaneously autoclaved. After cooling, a glass electrode, previously sterilized with 0.1 per cent HgCl_2 and washed with sterile water, is inserted into each vessel. The vessels are placed in the shielded incubator at 38°C. The salt bridges are placed in proper position, and the electrodes are joined to their proper binding posts. The stream of gas is started and the potential of each electrode is read and recorded every 10 minutes. The medium is electrolyzed if necessary to adjust the potentials, and acid or alkali is added to adjust the pH to the previously chosen levels. The brain-broth-culture inoculum, 0.2 ml., is added to each vessel, and after one or two more readings, the dye is added, 2 ml. of 0.03 per cent toluylene blue. In the next 12 to 24 hours, readings are taken on the platinum electrodes in the experimental vessels at least once every 10 minutes; and on all glass electrodes and on the platinum electrodes in the control vessels, once every half hour. Depending upon the relation between the observed and the desired potential, electrolysis is administered so as to keep the potential constantly at the desired level within a range of 10

to 20 millivolts. During the early stages of an experiment, little electrolysis is needed, but while vigorous growth is taking place, as much as 15 milliamperes of positive electrolysis for half the time (5 out of every 10 minutes) may be necessary to counteract the reducing tendency of the growing culture, so that the potential remains constant. Care is taken to insure a continuous flow of gas at a rate of one bubble per second. The first indication of growth is an increase in reducing power, and this is followed within an hour or two by a noticeable turbidity, which becomes marked in 2 to 5 additional hours. The time of the first definite appearance of turbidity is called the time of beginning of growth.

DATA ON LIMITING POTENTIAL STUDIES

Studies on Bacteroides Vulgatus

Table I gives the results of four experiments on the relation of electrolytically maintained oxidation-reduction potential and growth in anaerobic cultures of *Bacteroides vulgatus*. Each experiment is divided into periods of 2 to 4 hours, and the average potentials read every 10 minutes for each of these periods, as well as for the entire experiments are calculated. The first three experiments show that at average potentials of +0.135, 0.138, and 0.144, growth consistently occurred in from 10 to 15 hours; whereas at an average potential of +0.158 (Experiment 4) there was no growth in 20 hours. The control cultures with an Eh of about -0.150 showed growth in 8½ to 10½ hours.

In the last two columns of Tables I and II are given the coulombs of electrolysis (calculated from milliamperes and seconds duration) which must be administered in order to keep the Eh constant, and this is a measure of the reducing power of the culture. It will be noted that whenever growth occurs, indicated as + turbidity, there is an increase in the coulombs of positive electrolysis, usually even in the periods immediately preceding the first noticeable turbidity, indicating an increase in reducing power during growth. This is more strikingly shown in Table II with *Cl. sporogenes*.

Studies on Cl. sporogenes

Table II gives the results of three experiments showing the relation between electrolytically maintained oxidation-reduction potential and growth in anaerobic cultures of *Cl. sporogenes*. It is seen that at an average Eh of 0.136 volt there is growth in 13 hours, while at 0.164

there is no growth in 18 hours. In the third experiment there was no growth at an average Eh of 0.155 in 9 hours, but when later the potential

TABLE I

Effect of Electrolytic Control of Potential on Growth of Bacteroides vulgatus in the Presence of Nitrogen

Average Eh	Average deviation	Time interval	pH	Turbidity	Electrolysis in coulombs
	mv.	hours			
128	15	4	6.93 to 6.39	—	1.5
144	4	4	6.39 to 6.08	—	4.4
130	7	2½	6.08 to 5.87	+	1.6
Total	135	10½			
145	7	4	6.65 to 6.50	—	-0.65
138	5	4	6.50 to 6.46	—	-1.3
130	6	2½	6.46 to 6.30	+	3.5
Total	138	10½			
141	10	3	5.87 to 6.70	—	1.9
143	5	3	6.70 to 6.60	—	3.8
146	2	3	6.60 to 6.37	—	4.5
144	3	3	6.37 to 6.02	+	4.5
146	3	3	6.02 to 5.68	+	6.5
Total	144	15			
161	1	4	6.68 to 6.63	—	4.7
156	2	4	6.63 to 6.47	—	2.1
157	2	4	6.47 to 6.43	—	2.6
155	4	4	6.43 to 6.33	—	3.8
158	12	4	6.33 to 6.28	—	3.3
138*	3	4½*	6.28 to 6.22	—	1.3
Total	158	20			

* Potential lowered to determine viability of culture. Not included in final average potential.

Controls showed turbidity and acid formation 8½ to 10½ hours; final pH 4.8 in 22 to 24 hours; potentials reach Eh -0.150 to -0.175.

was put at 0.128 for 4 hours, growth occurred. Control cultures (without electrolysis) showed growth in from 6½ to 8 hours with an average

Eh of -0.110 . The limiting potential for growth of *Cl. sporogenes* at an initial pH of 6.6 is thus seen to be the same as that of *Bacteroides vulgatus*, namely, about 0.150 volt.

TABLE II

Effect of Electrolytic Control of Potential on Growth of Clostridium sporogenes in the Presence of Nitrogen

Average Eh	Average deviation	Time interval	pH	Turbidity	Electrolysis in coulombs
mv.	mv.	hours			
133	6	3	6.78 to 6.63	—	0.57
138	5	3	6.63 to 6.60	—	0.64
134	4	3	6.60 to 6.23	—	4.4
140	18	4	6.23 to 5.28	+	18.6
Total	136	13			
161	13	4	6.53 to 6.33	—	-0.3
164	1	4	6.33 to 6.28	—	0.7
166	1	4	6.28 to 6.23	—	0.5
165	1	6	6.23 to 6.05	—	1.1
Total	164	18			
157	7	3	6.72 to 6.62	—	0.56
156	3	3	6.62 to 6.59	—	0.58
151	4	3	6.59 to 6.22	—	4.8
128*	8	4 ¹	6.22 to 5.30	+	20.4
Total	155	9			

* Potential lowered to determine viability of culture. Not included in final average potential.

Effect of pH on the Limiting Potential

In the preceding studies the pH of the medium gradually fell as growth and positive electrolysis occurred. In order to study the effect of pH on the limiting potential, alkali was added as needed so that the pH was kept approximately constant, that is within a range of 0.2 pH. The immediate effect of alkali addition, and corresponding rise in pH was to cause a more negative Eh; the addition of acid and fall in pH had the reverse effect; and these effects occurred even in the absence of dye.

The effect of acid or alkali addition on the Eh was about the same as on the glass electrode potential. Electrolysis, however, affected the Eh much more (about 5 to 10 times) than it did the glass electrode potential and so a judicious choice of positive electrolysis and alkali addition allowed a reasonably good control of both pH and Eh.

Three pH levels were studied: 6.6, 6.2, and 7.0, using *Cl. sporogenes*, under anaerobic conditions, with electrolytic control of potential. The data (reported in detail in a Ph.D. dissertation, Katz, 1941 [15]) may be summarized thus: the limiting potentials for growth at pH 6.2 (0.139 volt) and at pH 7.0 (0.132 volt) are definitely more negative than at pH 6.6 (0.148 volt). It seems probable that the limiting potential has a maximum at pH 6.6, but more data are needed to prove this.

GROWTH OF OBLIGATE ANAEROBES IN THE PRESENCE OF AIR

Vennesland and Hancic (5) showed that the anaerobe *Bacteroides vulgatus* would grow in air-nitrogen mixtures, provided the oxidation-reduction potential was kept below +0.150 volt, but growth was never observed when the proportion of air to N₂ exceeded 40%, that is, about 8% O₂. It was of interest to determine whether obligate anaerobes can grow in a continuous current of undiluted air when the potential is maintained by negative electrolysis below the limiting potential for growth. Since negative electrolysis involves alkali formation, the electrode vessels were fitted with burettes containing 0.5 N HCl which was added at intervals whenever the pH rose above 7. The rate of flow of air varied from one bubble per second to one bubble every 4 seconds, and these variations made necessary corresponding variations in the amount of electrolysis necessary to keep the potential constant.

In Experiment 1 of Table III with *Cl. sporogenes* nearly the maximum amount of negative electrolysis (about 10 milliamperes for half of the time) was used, and growth occurred in a continuous current of air at an average Eh +0.082. In Experiment 2 of Table III, where less negative electrolysis was used so that the average potential was 0.124, growth did not occur. Table IV shows similar results with *Bacteroides vulgatus*, where there is growth at an average potential of +0.129 but no growth at +0.143. These experiments show that growth of these anaerobes does occur in a continuous current of air, when the potential is maintained sufficiently negative. At first sight they seem to indicate that the limiting potential for growth in air is slightly more negative than it is anaerobically or at low O₂ tensions. However, because of the

TABLE III

Growth of Clostridium sporogenes in Air with Negative Electrolysis

Average Eh	Average pH	Time interval	Turbidity	Electrolysis in coulombs	HCl m.-eq. per 75 ml.
		hours			
95	6.6	4	—	—66.9	0.5
93	6.2	4	—	—66.0	1.2
57	6.4	4	+	—39.0	
Total . . . 82	6.4	12			2.6
129	6.4	4	—	—11.6	0
135	6.9	4	—	—12.0	0
131	7.1	4	—	—6.0	0
140	7.3	4	—	—5.9	0
104	6.2	4	—	—18.3	0.5
Total 124	6.8	20			0.5

TABLE IV

Growth of Bacteroides vulgatus in Air with Negative Electrolysis

Average Eh	Average pH	Time interval	Turbidity	Electrolysis in coulombs	Acid in m.-eq. per 75 ml.
mv.		hours			
145	6.7	4	—	—56.1	0.20
148	6.8	4	—	—13.5	0.10
144	6.7	4	—	—29.2	0.42
137	6.7	4	—	—67.4	0.16
142	6.6	4	—	—46.4	0.14
Total . 143	6.7	20			
136	6.8	4	—	—61.5	0.76
133	6.9	4	—	—34.9	0.24
117	6.8	4	+	—34.6	0.52
97*	6.7*	4*	+	—72.9	0.60
97*	6.8*	4*	++	—49.9	0.27
Total..... 129	6.8	12			

* Values not included in final average potential.

violent opposition of two powerful forces, the oxidizing effect of air and the reducing effect of negative electrolysis, the potential and pH could not be well controlled. The pH fluctuated by as much as 1 unit and the oxidation-reduction potentials by as much as 80 millivolts; and the only conclusion which can be fairly drawn is that the limiting potential in the presence of air is approximately the same as under anaerobic conditions.

STUDIES ON O₂ CONSUMPTION OF OBLIGATE ANAEROBES

Katz and Hanke (13) have reported that when *Bacteroides vulgatus* grows in the presence of O₂, it is consumed with a minimal Q_{O₂} of 11. In these experiments, gas mixtures containing 3 to 9 per cent O₂ were continuously circulated from a closed system through a culture medium containing shiny platinum electrodes for reading oxidation-reduction potentials so that conditions for growth could be accurately known and controlled; and the O₂ content was measured at regular intervals by sampling and analyzing the gas mixture. These observations are here confirmed and extended.

In these experiments no dye was added and the culture was not electrolyzed; so the O₂ consumption cannot be related to any catalytic effect of the dye, or to any reducing action of electrolysis. The conditions for growth or no growth are controlled only by the rate at which the O₂-containing gas mixture is circulated through the culture: a slow rate allows a more negative potential and thus permits growth; a more rapid rate makes the potential more positive and thus prevents growth. The relation of O₂ consumption and growth is made very convincing by the observations that whenever there is growth (determined by low potential) in the presence of O₂, there is also O₂ consumption; whenever there is no growth (determined by high potential) at exactly the same composition of medium, inoculum, and O₂ tension, then there is no O₂ consumption.

Table V summarizes the results of O₂ consumption studies. The first four experiments are at potentials more negative than 0.150 volt where turbidity develops, the pH falls, and O₂ consumption occurs. The last two are at potentials above 0.150 volt and here with the same medium and the same inoculum there is no turbidity, no pH change, and no O₂ consumption. The average O₂ consumption was 0.17 ml. O₂ per hour, by 75 ml. culture medium. At the end of four experiments where growth occurred the medium was centrifuged and the residue

washed with water in weighed centrifuge tubes. The average dry weight of the organisms was 15 mg. The Q_{O_2} is thus 11. *

It is of interest to compare the chemical equivalents of O_2 consumed by the growing culture with the electrical equivalents of positive electrolysis which are needed to keep a growing culture at constant potential. Since 22.4 liters of O_2 is 4 equivalents, 0.17 ml. is 30 microequivalents, and this is the average O_2 consumed by the growing culture of *Bacteroides vulgatus* per hour. Now from Table I the positive electrolysis needed to keep the growing culture of *Bacteroides vulgatus* at

TABLE V

O₂ Consumption Related to Growth of Bacteroides vulgatus at Controlled Oxidation-Reduction Potential

Average Eh	pH		Time	Per cent O_2 in gas		ml. Pure O_2 consumed by culture	
	Initial	Final		Initial	Final	In time interval	per hr.
mr.			hours				
117	6.5	4.8	13	6.79	5.63	1.43	0.11
110	5.5	5.3	2.5	3.46	3.15	0.81	0.32
73	5.4	5.1	3	5.26	4.78	0.77	0.25
120	5.6	4.6	13	7.80	6.48	0.77	0.06
222	6.4	6.3	14.5	9.41	9.35	0.04	0.00
168	6.5	6.5	8.5	4.54	4.61	(+0.13)	(+0.01)

The volume of gas in the closed system containing the culture medium varied from 250 ml. at the beginning to 50 ml. at the end. The decrease is due to the samples which were withdrawn for analysis.

constant potential varied from 0.7 to 2.2 coulombs per hour. It is likely that the higher coulomb figure realized in the later stages of growth after the third hour is more nearly comparable with the O_2 consumption value, since the O_2 consumption figures were all in prolonged experiments. Since 96,500 coulombs is one equivalent, 2.2 coulombs is 23 micro-equivalents. The correspondence between 30 micro-equivalents of O_2 and 23 micro-equivalents of positive electrolysis is as close as could be expected, and indicates by each of these two methods of measurement that the reducing power of this anaerobe is the same. From Table II, it is seen that the reducing power of an actively growing culture of *Cl. sporogenes* on the same nutrient broth

medium is about 5 coulombs per hour, and this is more than twice as great as that of *Bacteroides vulgatus*; its O_2 consumption was not measured.

DISCUSSION

The significance of a limiting oxidation-reduction potential for growth of obligate anaerobes and its place in a theory of anaerobiosis has been discussed by Vennesland and Hanke (5). We believe that the growth of these obligate anaerobes depends upon enzymes which have reversibly oxidizable and reducible groups; that these enzymes are active only in the reduced form; and that the reversible oxidation and reduction of these groups occurs for a given pH at a certain level of oxidation-reduction potential. The separation of these enzymes from the bacterial cells, and the demonstration of the relation of their activity to the oxidation-reduction potential of the solution, are problems for the future. Of interest here is that the limiting potential which Sizer and Tytell (9) report for urease, is the same, 0.150 volt at pH 6.8, as that which we found for the growth of these obligate anaerobes. It is also very close to the Eh of 0.160 volt which Lipmann (16) reported caused a marked inhibition of the rate of fermentation of yeast maceration juice. These potentials are, however, much more negative than the limiting potentials reported by Reiss for papain (8), by Sizer (10) for phosphatase, and also more negative than the potential levels which must be involved in the inhibition of the action of carbonic anhydrase (Kiese and Hastings [7]).

Preliminary unpublished experiments show that other obligate anaerobes do not have the same limiting potentials for growth as those found for the two organisms studied here. For *Cl. welchii* and *Cl. histolyticum* we find limiting Eh values of 0.165 and 0.090, respectively, at pH 6.6. The more negative limit for *Cl. histolyticum* may be related to the fact that in our medium *Cl. histolyticum* is an alkali former while the other three, *Cl. welchii*, *Cl. sporogenes*, and *Bacteroides vulgatus*, are acid formers.

The fact that the limiting potential for growth remains the same, while the O_2 tensions which the organisms will tolerate vary widely, indicates that the limiting factor is Eh and not O_2 tension. For instance, immediately after inoculation of a medium with an obligate anaerobe, as little as 0.1 per cent O_2 is enough to prevent all growth. But after growth is well started in the absence of O_2 , the organisms will tolerate

and continue to grow in an atmosphere of 5 to 8 per cent O_2 . And with adequate negative electrolysis the organism will grow in a current of undiluted air. The constant factor distinguishing growth from no growth at all these O_2 tensions is the Eh level: immediately after inoculation when the reducing power of the culture is very little, 0.1 per cent O_2 is enough to keep the Eh more positive than 0.150 volt and so there is no growth; after growth is well started, 5 per cent O_2 is not enough to counteract the vigorous reducing power of the growing organisms, not enough to keep the potential more positive than 0.150 volt, and therefore growth continues. We conclude that O_2 *per se* has little if anything to do with the prevention of growth of obligate anaerobes; it influences their growth only to the extent that it affects the oxidation-reduction potential. The fact that the same limiting potential for growth is found in the presence of a reversible oxidation-reduction indicator (toluylene blue) as is found without such dye adds confidence to the validity of the figure.

The observations on O_2 consumption are, we believe, the first to show that obligate anaerobes consume O_2 during growth. This is particularly interesting in view of the discussion by Broh-Kahn and Mirsky (17) to the effect that there is no evidence that obligate anaerobes ever consume oxygen, and that strict anaerobes contain no catalysts for the activation of oxygen. Warburg and Christian (18) have shown that certain anaerobes have in fact the highest concentration of yellow enzyme found in any cells. If the oxygen consumption observed in the anaerobes in our studies is catalyzed by the yellow enzyme, it would involve production of hydrogen peroxide. Vennesland and Hanke (5) were unable to demonstrate hydrogen peroxide in cultures of *Bacteroides vulgatus*, exposed to air. On the other hand, Sherman (19) showed that some anaerobes contain catalase, so the absence of peroxide would follow if catalase is present in the *Bacteroides* culture.

The Q_{O_2} of 11 is a minimal value. This is very close to the Q_{O_2} of 6 to 12 calculated by Stickland (20) from observations on methylene blue reduction in a Thunberg tube by *C. sporogenes*. It is of the same order of magnitude as the values (3 to 20) reported by Krebs (21) for resting mammalian tissue. For aerobic micro-organisms, values of from 13 to 150 are reported (22). When we recognize that our value of 11 is a minimal value based on the final weight of organisms, and that the actual Q_{O_2} based on the average weight of organisms would probably

be about twice this value, we see that the Q_{O_2} of anaerobes is of the same order of magnitude as that of aerobes.

The electrolytic method of controlling oxidation-reduction potential is recommended whenever it is desired to maintain a constant level of potential in the face of the variable addition or removal of oxidizing or reducing agents; or whenever carefully graded quantities of oxidizing or reducing agent are to be administered *without the addition of a foreign substance*; that is, by the mere oxidation of such reducing agents, or the reduction of such oxidizing agents, as are already present in the solution. It should also be useful in electrometric titrations, where, of course, the added oxidizing or reducing agent is not a new substance from a burette, but simply a measured number of coulombs of electric current. It should be of especial value in biological investigations.

SUMMARY

1. An electrolytic method for controlling the oxidation-reduction potential of aqueous solutions is described, by which it is possible to maintain constant the Eh of bacterial cultures without the addition of any foreign agents to the medium.

2. From studies at a variety of potentials and O_2 tensions of cultures of *Bacteroides vulgatus* and *Cl. sporogenes*, it is found that the limiting Eh for the initiation of growth of these obligate anaerobes is 0.150 volt at pH 6.6. At pH 7.0 and at pH 6.2 the limiting Eh is more negative by about 15 millivolts.

3. When the potential is kept sufficiently negative electrolytically, these anaerobes will grow in a continuous current of air.

4. Since these limiting potentials are independent of marked variations in O_2 tension, we feel that the limiting factor in the growth of these anaerobes is the Eh level and not O_2 tension.

5. Studies on O_2 consumption at controlled oxidation-reduction potential have shown that when *Bacteroides vulgatus* grows in the presence of O_2 , O_2 is consumed with a minimum Q_{O_2} of 11.

6. The reducing power of an actively growing culture of *Bacteroides vulgatus*, about 30 micro-equivalents per hour (per 15 mg. dry weight of organisms in 75 ml. of culture medium), is approximately the same, whether measured by O_2 consumption or by the coulombs of positive electrolysis needed to keep the Eh constant.

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Changes in Vitamin Content during the Life of the Worker Honey-bee¹

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INTRODUCTION

In spite of the fact that insects serve as important sources of food for birds and numerous other animals including man in many countries, comparatively few investigations of their vitamin content have been made. None of the workers tried to follow the changes in the vitamin content during the life of any insect.

Giroud and Rakoto-Ratsimamanga (4) determined the amount of ascorbic acid in *Dytiscus marginalis*. Wollmann and co-workers (19) found that cockroaches, after 15 years on a sterile diet (46-47 generations) contained the same amount of ascorbic acid as specimens caught in the laboratory (13 mg./g. and 15 mg./g. for the experimental, and 10 mg./g. and 15 mg./g. for the wild males and females respectively). The investigators concluded that cockroaches can synthesize ascorbic acid. However, Nespor and Wenig (11) failed to find ascorbic acid in any of the insects they investigated (*Tenebrio molitor*, *Dytiscus marginalis*). Gourevitch (6) established the presence of variable amounts of unspecified flavin in several species of mature and immature insects. Koyanagi (9) and Sumi and Tuzuki (16) found riboflavin in silkworm pupae, while Drilhon and Busnel (2) discovered unspecified flavin, often in considerable amounts (80 to 2150 micrograms per gram of fresh matter), in the Malpighian tubules of various orders of insects. The presence of carotene, the precursor of vitamin A, has also been established (10, 12) in many insects. Joly (8) could not find vitamins A or D in the blood of a termite queen, but vitamins B₁, B₂, and C were present (25, 0.2-0.3, and 7 micrograms per one cubic centimeter of blood respectively). Recently Woods and co-workers (20) determined the "B" vitamin content of the whole red ants, cockroaches, termites, and *Drosophila* larvae. For comparison these values are included in Table I.

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EXPERIMENTAL

During the summers of 1941 and 1942 samples of bees and larvae were secured from colonies in the University of Minnesota apiary. The larvae were removed

TABLE I
Vitamin Content of Worker Honey-Bees
(Microgram per Gram of Fresh Matter)

Bees or parts of bees	Thi- amin	Ribo- flavin	Pyri- doxin	Nico- tinic acid	Panto- thenic acid	Ascor- bic acid
<i>Larvae</i>						
Young (1-3 days)	4.6	32.2	14.6	77.2	180.2	236
Sealed (old)	3.2	7.5	13.1	29.9	18.7	87
<i>Pupae</i>						
Young (1-2 days)	5.8	5.6	9.3	29.6	22.2	132
Old	3.5	8.4	7.3	33.5	25.2	63
<i>Emerging Bees</i>						
Whole	4.7	9.0	6.2	37.0	20.3	43
Heads.	10.8	17.4	12.5	34.8	27.9	302
Digestive tracts. . .	9	8.9	6.0	13.4	5.4	0
Abdomens (without digestive tracts)	5.4	12.5	7.1	27.8	16.7	110
Thoraces	9.3	1.8	7.2	80.0	37.2	5
<i>Adult bees</i>						
Whole	7.7	8.7	8.2	40.8	14.3	188
Heads	12.2	14.4	15.2	71.8	25.1	492
Digestive tracts	3.9	9.7	9.6	29.2	9.7	607
Abdomens (without digestive tracts). . .	9.2	22.7	8.4	60.5	22.7	8
Thoraces	8.9	8.0	6.5	47.7	14.9	6
<i>Other insects (20)</i>						
Red ant (<i>Dolichoderus</i>)	3.2	6.08	.67	20.5	12.5	
Cockroach (<i>Periplaneta americana</i>)	4.4	7.11	1.3	33.0	17.5	
Termites (<i>Zootermopsis</i>)..	2.3	4.75	.32	32.0	16.0	
<i>Drosophila virilis</i> larvae						
N. Y.	4.2	8.11	1.3	36.5	20.0	
N. O. . .	4.4	8.22	.94	37.5	20.5	

from the cells and freed from the adherent food by rolling them over filter paper. The young bees were taken out of the cells of a comb at the time of emergence. Adult bees of an unknown age were used. The bees were killed with chloroform

and dissected. The larvae, the bees, and the parts of the latter were weighed immediately after obtaining them from the comb or dissecting.

The samples were macerated in 100 cc. of water and three aliquots were taken for vitamin determinations. The methods used were: For thiamin, that of Hennessy and Cerecedo (7); for riboflavin, that of Snell and Strong (13); for ascorbic acid, Bessey (1); for pantothenic acid, Strong, Feenoy, and Earle (15); for nicotinic acid, Snell and Wright (14).

The results of the determinations are presented in Table I.

DISCUSSION AND CONCLUSIONS

From the data presented in Table I it is evident that the concentration of the B vitamins in the whole bees is higher than the values reported for other insects. An examination of the table also shows that the parts containing glands have, on the whole, a greater vitamin content per gram of fresh matter than those consisting predominantly of muscles. Giroud and Rakoto-Ratsimamanga (4), studying the distribution of vitamin C in the invertebrates, found this to be true in *Dytiscus marginalis*, the sexual glands of which contained 0.47 mg./g. ascorbic acid while the muscles had only 0.06 mg./g. of fresh matter. Giroud and co-workers (5) found ascorbic acid present in all the animal tissues they analyzed. Moreover, according to these authors, the morphology and function of organs or tissues determines their proportion of this vitamin, the endocrine glands containing the highest concentrations. In a later paper Giroud and co-workers (3) presented further evidence of this relationship. They point out that muscular tissues, for instance, are the poorest in ascorbic acid, but their content differs according to function and structure. Thus the skeletal muscle of beef contains 0.016 mg., the cardiac muscle 0.038 mg., and the smooth muscle 0.063 mg. ascorbic acid per gram of fresh matter.

It would be of interest to compare the content of other water soluble vitamins in glands and muscles in order to ascertain whether the same relationship, as in the case of ascorbic acid, exists between them. Recently Wright and co-workers (21) published a study on the vitamin content of normal tissues. Taking in consideration only the averages for liver and muscles (Table II), one can state that glandular tissues of mammals have a larger content of most of the "B" vitamins than the muscular tissues. Closer examination of the table reveals that there are exceptions (thiamin in hog). The data presented by Taylor, *et al.* (17) show the same relationship between the liver and the skeletal muscles of humans.

In order to compare the distribution of vitamins in the vertebrates with our results the vitamin content of the larvae, pupae, heads, and

TABLE II
Vitamin Content of Animal Tissues
(Microgram per Gram of Dry Matter)

Kind of Tissues	Thi-amin	Ribo-flavin	Pyri-doxin	Nico-tinic acid	Panto-thenic acid	Ascor-bic acid
1. Bees						
<i>Larvae</i>						
Young (1-3 days)	22.9	161.3	73.2	393.6	916.1	1180
Scaled	13.9	32.8	56.8	130.4	81.6	380
<i>Pupae</i>						
Young (1-2 days)	26.4	25.3	42.5	131.3	101.0	598
Old	23.1	57.0	48.9	229.2	169.9	420
<i>Emerging bees</i>						
Heads	45.4	81.9	54.8	146.2	118.4	1270
Thoraxes	39.0	20.1	29.7	334.7	156.2	19
<i>Adult bees</i>						
Heads	40.1	47.4	50.2	238.2	85.3	1620
Thoraxes	26.3	23.6	19.3	142.1	44.6	16
2. Other animals (21)						
Rat liver	31.0	100.0	2.5	530	370	
Mouse liver	10.0	39.0	2.2	270	120	
Hog liver	6.4	71.0	5.6	390	160	
Average	15.8	70.0	3.4	397	217	
Rat muscle	3.1	10.0	1.2	310	32	
Mouse muscle	1.5	1.7	1.5	220	25	
Hog muscle	16.0	21.0	5.8	180	28	
Average	7.0	11.9	2.8	247	27	
Veal muscle (18)	11.4	12.1	17.3	568*	52.0	
Beef muscle (18)	7.5	9.0	15.4	269*	23.9	

* Found by computation from Waisman and Elvehjem (18).

thoraxes of the emerging and adult bees was recalculated per gram of dry matter (Table II).

From the table it is evident that heads (containing glandular tissues) of the worker bees are richer in vitamins than the thoraxes (muscular

tissues). However, some discrepancy is also manifested, the nicotinic and pantothenic acid contents of the heads of emerging bees being lower than that of the thoraces. In connection with this it should be mentioned that the pharyngeal glands in the heads of emerging bees are not active.

There are also changes in the vitamin content during the development of bees. In general, younger stadia have greater amounts of vitamins per unit of weight than the older ones. The amount of vitamins per gram of fresh or dry matter in the young larvae is considerably larger than that of the sealed larvae. This is not so evident in the pupae. It is necessary to note, however, that there are excessive histochemical changes in insects during the pupal stadium.

In comparing the changes in the vitamin content during the growth of the emerged worker bees one has to bear in mind that these changes in the heads would largely depend on the activity of the pharyngeal glands located in the head capsule. These glands are most active in the nurse bees (normally 5-10 days old) and usually, but not always, become functionless as the bees grow older. Since the exact age of the adult bees was not known in our experiments and consequently the physiological activity of the glands could not be estimated, the results of the comparison might not represent the actual relation between the vitamin content of the young and the old specimens. Therefore it seems that the use of the thoraces would be more indicative of the changes occurring during the growth of the bees. With the exception of riboflavin, the vitamin content of the thoraces of young bees is also higher than that of the old.

The concentration of vitamins in the abdomens from which digestive tracts have been removed is lower in the emerging bees. This phenomenon can be explained by the fact that the so called "fat body" of the abdomen serves as a general reserve tissue and consequently its vitamin content is likely to be greater when the stores of reserve materials begin to accumulate later in life. There are also glands present in the abdomens the activity of which may be greater in the older bees.

The digestive tracts of the older bees are richer in vitamins than those of the emerging bees. This is not surprising because the digestive tracts of the latter are free of any remnants of food since the larvae defecate before changing to pupae. The rectum of an emerging bee is full of a water-clear liquid, while that of an older bee contains excreta and the midintestine includes ingested food. Therefore the concentration of vitamins in the digestive tracts of the adult bees may to a great extent

depend on the type and the amount of food eaten and the accumulation of feces in the rectum. To what extent the Malpighian tubules, which are connected with the digestive tract, influence the vitamin changes in the bees cannot be ascertained from the present data. Judging from the literature (2), the abundance of ascorbic acid in the digestive tracts of adult bees can also be explained by a greater concentration of this vitamin in the Malpighian tubules of the insect.

For comparison, summarized averages of the vitamin content of the muscles of beef and veal, taken from Waisman and Elvehjem (18), are presented in Table II. The table reveals that, for the samples investigated, as in the case of worker bees, the vitamin content of the dry matter of muscles of younger cattle is greater than that of the older ones.

It is also evident that the vitamin content of the dry matter of the muscles of bees, except for nicotinic acid, is two to three times greater than that of the cattle. Giroud and Rakoto-Ratsimamanga (4) established the fact that the muscles of invertebrates contain three times as much ascorbic acid as those of vertebrates. This also appears to be true in relation to the majority of other vitamins.

SUMMARY

The thiamin, riboflavin, pyridoxin, nicotinic, pantothenic, and ascorbic acids contents of the young and old (sealed) larvae, young and old pupae, emerging and older worker bees, and their parts were determined. On the whole, the vitamin content of heads (containing glandular tissues) is greater than that of the thoraces (muscular tissues) of worker bees. Younger stadia seem to have more vitamins per unit of fresh or dry weight than the older stadia. The vitamin content of the thoracic muscles of bees, except for nicotinic acid, is two to three times greater than that of some vertebrates.

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The Dispersion of Keratins

I. Studies on the Dispersion and Degradation of Certain Keratins by Sodium Sulfide

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INTRODUCTION

Keratin materials, such as feathers, hoofs, and horns, are available in large quantities and possess several properties that suggest suitability for utilitarian purposes. Among these properties are fibrous or horny texture, elasticity, high tensile strength, and a marked resistance to chemical and bacterial attack. However, the general insolubility of keratins in comparison with most other proteins is a serious handicap in certain types of industrial utilization.

The purpose of one phase of work undertaken in this laboratory was the investigation of methods whereby keratins might be dispersed in media from which the protein could be recovered in a more tractable form than that of the original keratins. Although, in such methods, maximum dispersion of keratin and maximum yield of product would be desirable, it would also be advantageous to retain in the final protein as many as possible of the desirable characteristics of the original keratin materials. The fulfillment of these conditions obviously requires a method for dispersing keratin with a minimum of chemical degradation.

A survey of the literature (1-41) showed that numerous methods have been employed for the dispersion of keratins. These methods involve varying degrees of protein degradation. Indeed, the splitting of the keratin molecules into smaller units seems to be a prerequisite for dispersion by any means studied thus far.

Of the dispersion methods, the use of reducing agents in alkaline solution seemed most likely to yield a rapid and efficient method for the dispersion of keratin with minimal change in chemical constitution and physical properties. Sodium sulfide was selected for use in initial investigations, the results of which are reported in this paper.

Goddard and Michaelis (19) studied in some detail the dispersion of the keratins of wool and chicken feathers in alkaline thioglycolate, cyanide, and sulfide, and also the properties of the precipitates obtained by acidification of the alkaline dispersions. These authors proposed the hypothesis that for the dispersion of keratins not only must the disulfide groups be split by a reducing agent, but also the salt linkages of the keratin molecule must be broken by alkali, thus explaining the inability of reducing agents to disperse keratins in solutions less alkaline than pH 10. Although Goddard and Michaelis suggested that the alkaline action is a prerequisite for the reduction, Harris and co-workers (42) have more recently shown that the disulfide groups of wool can be reduced by thioglycolic acid over a wide pH range. No dispersion occurs if the reduction is carried out in neutral or acid solution, but the alkali-solubility of the reduced wool is much greater than that of untreated wool.

From this previous work it seems that reduction of the disulfide groups and alkaline cleavage of the salt linkages (and other secondary linkages) are mutually independent reactions, both of which, however, are necessary for dispersion of keratins. Although the rôle of the reducing ions present in a sodium sulfide solution may be limited to reduction of the disulfide linkages in the keratin molecule, the extent to which the rôle of the hydroxyl ions is restricted to secondary-bond cleavage obviously depends upon the conditions used for dispersion of the keratin. For example, hydrolysis of peptide linkages would be favored by high concentrations of sodium sulfide, high temperatures, or prolonged treatment. Side-reactions may also occur under certain conditions, since it has been shown that alkalies (including sodium sulfide) can bring about conversion of cystine to lanthionine (15, 43) and destruction of the hydroxy amino acid residues (44) in keratins and other proteins. Determination of conditions under which dispersion might be effected with a minimum of hydrolytic degradation and side-reactions seemed desirable. A study was made, therefore, of the influence of various factors upon the degree of dispersion and the extent of degradation of feather keratin by sodium sulfide.

Application to other keratins of the conditions found to be optimal for dispersion of feather keratin revealed noteworthy differences between individual keratins with respect to dispersibility and stability in sodium sulfide solutions. The keratins of hoof, hog hair, and wool were found to behave differently from the keratin of feathers and to show somewhat less variation in behavior among themselves.

EXPERIMENTAL

Chicken Feather Keratin

The feathers used in these investigations were whole body feathers from mixed breeds of chickens, obtained from a killing plant on the day they were picked. After the feathers were washed several times in cold water, they were spun in a basket centrifuge and were air-dried at room temperature. Fat-soluble material was then removed by extraction with benzene for at least 15 hours in large Soxhlet extractors. Although several lots of feathers were used in these studies, the keratin used in any one series of experiments was from a single lot. The nitrogen content of the extracted material varied from one lot to another (15.7–16.1 per cent on moisture-free basis). The moisture content varied between 7 and 10 per cent, depending on the atmospheric humidity; the relationship between water content of feathers and humidity of the air has been reported by Wilson and Fuwa (45). No difference in behavior was observed between dry-picked and wet-picked (scalded) feathers or between whole feathers and feathers which had been ground in a Wiley mill equipped with a 2-mm. screen.

The sodium sulfide used in these studies was reagent-grade crystalline $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$. Dispersion of the keratin was carried out in stoppered Erlenmeyer flasks suspended in a constant temperature bath. The flasks were shaken by hand at 15-minute intervals. Each dispersion was filtered through a Buchner funnel after addition of Hyflo Filter Aid (manufactured by Johns-Manville Corporation) in an amount equal to 80 per cent of the weight of the original keratin.

Extent of dispersion was measured either by the weight of undispersed residue or by the amount of total nitrogen dispersed. In the first method, the undispersed material, with the added filter aid, was washed several times with water while on the funnel and was dried at 103°C. overnight; the moisture content was then allowed to come to equilibrium with the air at room temperature for 24 hours before weighing. In the second method, the total nitrogen dispersed was calculated, from the volume of Na_2S solution used and Kjeldahl analyses of the filtered dispersion, as per cent of the total nitrogen in the original keratin sample.¹ In sev-

¹ The nitrogen determinations were carried out by A. Bevenue, of this laboratory.

eral experiments in which both methods were used, results agreed within 3 per cent.

Extent of protein degradation was estimated by the following method. Preliminary experiments had shown that maximal precipitation of the dispersed protein occurred at pH 4.2. An aliquot of the filtered dispersion was acidified to this pH by dropwise addition of glacial acetic acid and was allowed to stand at room temperature for one hour before removal of the precipitate. The acid-precipitated fraction was then determined either by weighing the washed and dried precipitate or by calculating the difference between the nitrogen content of a filtrate of the acidified dispersion and that of the original dispersion. In the experiments in which the precipitates were weighed, they were removed by centrifuging and ground in a mortar with successive portions of water, acetone, and ether; the products were finally dried *in vacuo* over CaCl_2 . The extent of decrease from the maximal amount of acid-precipitable protein was considered to be a measure of the protein degradation.

Influence of Na_2S Concentration on Extent of Dispersion. Several 5 g. portions of feather keratin were treated for 3 hours at 30°C . with 200 ml. of Na_2S solution at different concentrations, and extent of dispersion was measured by residue weight. Solutions of 0.5 M, 0.1 M, and 0.05 M Na_2S dispersed, respectively, 85, 84, and 78 per cent of the keratin. Only about 10 per cent of the keratin was dispersed by 0.025 M Na_2S .

Influence of Ratio of Keratin to Na_2S upon Extent of Dispersion and Yield of Acid-Precipitable Fraction. Several series of dispersions were prepared by treatment for 3 hours at 30°C . A different concentration of Na_2S was used for each series, and within each series the amount of keratin treated with a fixed volume of sulfide solution was varied. Extent of dispersion was measured by residue weight. Each filtrate, with the washings from the corresponding residue, was transferred immediately to a cellophane bag and was dialyzed against running tap water overnight. The acid-precipitable fraction was isolated and weighed as described above. Dialysis was omitted in subsequent experiments.

Typical data are presented in Table I. The results showed that a maximal yield (based on weight of original keratin) of acid-precipitated protein was obtained by use of a different ratio of keratin to volume of sulfide solution for each concentration of Na_2S . A correlation between sulfide concentration and maximal yield of acid-precipitable fraction was revealed when the yields were calculated on the basis of weight of Na_2S used (Fig. 1).

It is evident that when 0.1 M Na_2S and a ratio of 10 g. of keratin to 100 ml. of Na_2S solution were used, the yield of protein precipitated at pH 4.2 was nearly maximal, based either on weight of original keratin or on weight of Na_2S used. With a ratio higher than 7.5 g. to 100 ml., however, the time required for wetting the feathers and for filtration of the dispersion was considerably increased and control of dispersion time

was correspondingly less accurate. Treatment of 7.5 g. of keratin with 100 ml. of 0.1 M Na_2S was selected, therefore, as a suitable combination of sulfide concentration and keratin: Na_2S ratio for dispersing feather

TABLE I
Effect of Keratin: Na_2S Ratio at Different Concentrations of Na_2S on Extent of Dispersion and Yield of Acid-Precipitable Protein
(Digestion time, 3 hours; temperature, 30°C.)

Feathers per 100 ml. soln. g.	Feathers Dispersed per cent of total	Yield of Protein Precipitated at pH 4.2 per cent of total
	0.04 M Na_2S	
1.60	76	66
2.00	70	63
2.50	62	56
3.75	28	20
6.25	13	9
7.50	11	6
	0.06 M Na_2S	
1.25	81	68
2.50	80	76
3.75	76	76
5.00	71	72
6.25	58	60
7.50	—	45
	0.1 M Na_2S	
1.25	84	60
2.50	82	68
3.75	83	73
5.00	80	74
6.25	79	76
7.50	79	76
8.75	80	77
10.00	78	75
11.25	73	66
12.50	60	59

keratin from the standpoint of maximal yield of protein obtained by subsequent precipitation.

Effect of Temperature on Extent of Dispersion. The curves presented in Fig. 2 were obtained from nitrogen analyses on representative samples withdrawn at various time intervals from the dispersion mixtures. That the higher degree of dispersion at 60°C. is due to increased hydrolysis

is suggested by the fact that only 88 per cent of the dispersed protein was precipitated at pH 4.2 from a dispersion prepared at 60°C. for 30 minutes, whereas a 97 per cent yield was obtained after treatment for 2 hours at 30°C. A slight odor of ammonia could first be detected in dispersions at the end of 10–12 hours; the nitrogen values obtained after

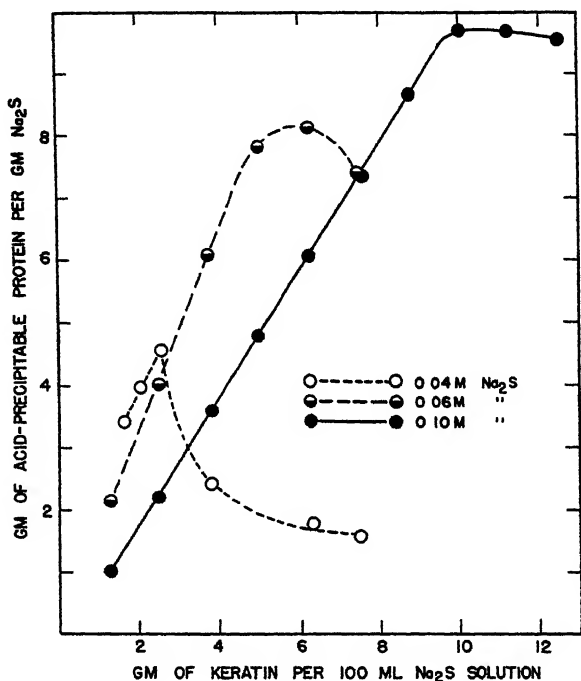


FIG. 1

Relationship Between the Keratin:Na₂S Ratio and the Yield of Acid-Precipitable Protein per g. Na₂S, Calculated from Data in Table I

this time, therefore, probably indicate a slightly lower extent of dispersion than that which actually occurred.

Effect of pH on Extent of Dispersion. Increasing amounts of approximately 0.2 N HCl, as indicated in Table II, were added, with shaking, to 20 ml. of 0.5 M Na₂S in each of eight 100 ml. volumetric flasks. The solutions were diluted to volume and the pH of each was determined. Each solution was then transferred to an Erlenmeyer flask containing

2.5 g. of feather keratin. The flasks were stoppered and set in a constant temperature bath at 30°C. for 22 hours; during the first 3 hours they were shaken at 15-minute intervals. At the end of the 22-hour

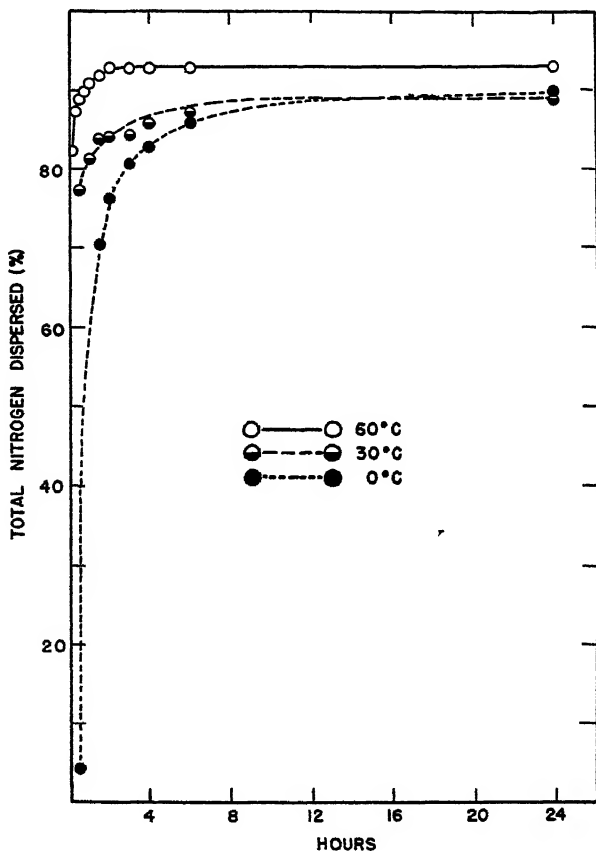


FIG. 2

Effect of Temperature on Rate of Dispersion
7.5 g. feather keratin per 100 ml. 0.1 M Na_2S .

period the pH of each dispersion mixture was determined and the mixtures were filtered. Extent of dispersion was determined by nitrogen analyses on the filtrates. The pH values and corresponding degrees of dispersion are presented in Table II.

The pH of dispersions may also be decreased by increasing the keratin:sulfide ratio. pH measurements were made in the experiments described above in connection with the effect of variation of the ratio of keratin to volume of Na_2S solution. As this ratio was increased, the pH of the dispersion mixtures gradually decreased. At the conditions under which the curves in Fig. 1 reached their maxima, the pH of the dispersion mixtures had dropped to 12. As the yields of acid precipitate per gram of Na_2S then decreased, the pH values became still lower.

Effect of Na_2S Concentration and Keratin: Volume Ratio on Extent of Dispersion. The curves presented in Fig. 3 show that extent of dispersion was increased by increasing the concentration of the Na_2S solution and also by decreasing the ratio of keratin to volume of

TABLE II

Effect of pH on Extent of Dispersion

(2.5 g. keratin per 100 ml. 0.1 M Na_2S at 30°C. for 22 hours)

HCl added ml.	Initial pH	Final pH	Dispersion per cent
None	12.6	12.5	90
25	12.3	12.1	87
43	11.8	10.9	53
50	10.9	9.8	3
51	9.9	9.5	3
52	8.4	9.0	1
56	7.9	8.2	1
76	6.8	7.0	1

Na_2S solution. Variations in either of these factors had no appreciable effect on the rate of dispersion until after the first hour of digestion.

Effect of Digestion Time on Extent of Dispersion and Yield of Acid-Precipitable Fraction. Each of five 7.5 g. portions of feather keratin was digested with 100 ml. of 0.1 M Na_2S at 30°C. After 50 minutes one digestion was stopped, and after each of four 30-minute intervals thereafter another digestion was stopped, and extent of dispersion and acid-precipitable fraction were determined. Comparison of the results, presented in Table III, indicated that optimal dispersion with minimal degradation was attained in about 2 hours. Examination of the corresponding time curve in Fig. 3 showed that after 1 hour and 50 minutes the rate of dispersion had dropped to 1.5 per cent of the original keratin per hour. Three dispersions were then prepared by treating 7.5 g.

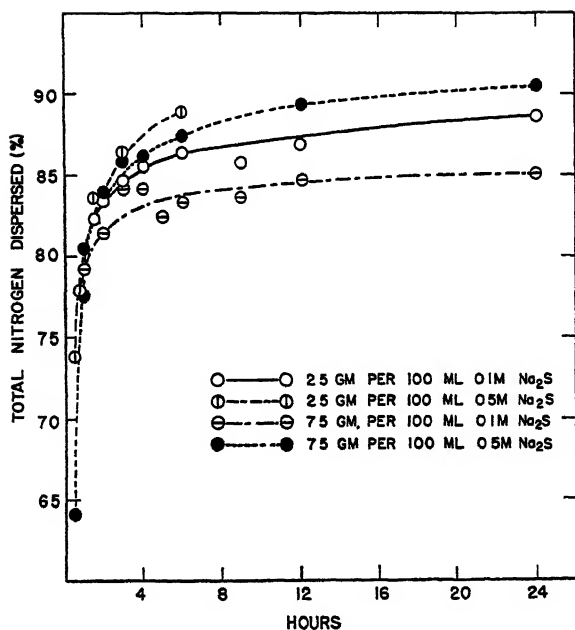


FIG. 3

Effect of Na_2S Concentration and Ratio of g. Feather Keratin to ml. Na_2S Solution on Rate of Dispersion

TABLE III

Extent of Dispersion and Yield of Acid-Precipitable Fraction As Influenced by Time of Digestion

(7.5 g. keratin per 100 ml. Na_2S solution at $30^\circ\text{C}.$)

Na_2S Concentration	Digestion time		Dispersed N per cent of total	N in Acid-Precipitable Fraction	
				per cent of total	per cent of dispersed
M	hr.	min.			
0.1	0	50	74	72	97
0.1	1	20	78	76	97
0.1	1	50	81	78	96
0.1	2	20	82	79	96
0.1	2	50	82	78	95
0.5	1	20	81	72	89
0.5	1	50	83	70	84
0.5	2	30	84	69	82

portions of keratin with 100 ml. of 0.5 M Na_2S at 30°C. One was treated for 1 hour and 50 minutes, another for 1 hour and 20 minutes, and the third for 2 hours and 30 minutes. These digestions are comparable with that treated for 1 hour and 50 minutes with 0.1 M Na_2S with regard, respectively, to time, per cent of keratin dispersed, and rate of dispersion. The results (Table III) indicated that the maximal yield of acid-precipitated protein from a 0.5 M Na_2S dispersion was less than that from a dispersion in 0.1 M Na_2S .

In the aforementioned experiments and in those described in the following section, the acid-precipitable fractions were separated from dispersions which had not been dialyzed. The amount of acetic acid required to adjust the pH to 4.2 varied with the concentration of sodium sulfide present. Separate experiments in which sodium acetate was added to sulfide dispersions showed a slight increase in the fraction precipitated at pH 4.2 as the concentration of sodium acetate was increased to 1.0 M . No attempt was made to incorporate in the results presented in this paper the effect of sodium acetate concentration on the solubility of the acid-precipitated protein. Introduction of such a factor would lower the yields of this fraction from dispersions in 0.5 M Na_2S one or two per cent below the observed yields reported here.

Comparison of Hoof, Hog Hair, and Wool Keratins with Feather Keratin

Fresh cattle hoofs and hog hair were obtained from an abbatoir. The hoofs were cleaned with cold water and were air-dried. They were then shredded with a coarse rasp and the filings were extracted with benzene. The hog hair was washed repeatedly in cold water and was then treated with a 1 per cent solution of pepsin at pH 1.5 for several days at 37°C. The pepsin-treated material was washed thoroughly with cold water, air-dried, and finally extracted with benzene. Raw wool was washed with cold water, air-dried, and extracted with benzene. It was again washed with water and air-dried, and extraneous material was removed by hand. All benzene extractions were carried out in Soxhlet extractors for at least 15 hours.

Extent of dispersion and yield of acid-precipitated protein were determined on dispersions of hoof, hog hair, and wool keratins prepared in 0.05 M , 0.1 M , and 0.5 M Na_2S solutions. The time, temperature, and ratio of keratin to volume of sulfide solution used in the preparation of these dispersions were those employed in the studies described by Goddard and Michaelis (19). The results, together with data on feather keratin dispersions prepared by the same methods (Table IV) show a marked difference in behavior between feather keratin and the other keratins. The feather keratin was more readily dispersed by 0.05 M and 0.1 M Na_2S and was more readily degraded by 0.5 M Na_2S .

A comparison was made between the dispersion rates of hoof, hog hair, and wool keratins in 0.1 *M* and 0.5 *M* Na₂S, using the ratio (7.5 g. of keratin per 100 ml. of Na₂S solution) which had been found to be more suitable for dispersion of feather keratin than the ratio used in the above-mentioned experiments. The results are presented in Fig. 4. Dispersions of feather, hoof, hog hair, and wool keratins were then prepared in 0.1 *M* and 0.5 *M* Na₂S; each digestion was stopped when the rate of dispersion had decreased to 1.5 per cent per hour (as calcu-

TABLE IV

Extent of Dispersion and Yield of Acid-Precipitable Fraction in 3-Hour Dispersions of Different Keratins

(2.5 g. keratin per 100 ml. 0.05 *M*, 0.1 *M*, and 0.5 *M* Na₂S at 30°C.)

Keratin	Dispersed N	N in Acid-Precipitable Fraction	
	<i>per cent of total</i>	<i>per cent of total</i>	<i>per cent of dispersed</i>
	0.05 M Na ₂ S		
Feather.....	78	76	96
Hoof.....	67	63	94
Hog hair.....	66	65	99
Wool.....	62	61	98
	0.1 M Na ₂ S		
Feather.....	84	75	90
Hoof.....	73	67	91
Hog hair.....	80	78	97
Wool.....	70	67	96
	0.5 M Na ₂ S		
Feather.....	85	57	66
Hoof.....	87	70	80
Hog hair.....	89	79	88
Wool.....	87	77	88

lated from Figs. 3 and 4). Extent of dispersion and yield of acid-precipitated protein were determined in each dispersion. The results (Table V) show that feather keratin was more rapidly dispersed by 0.1 *M* Na₂S than were the other keratins. At this Na₂S concentration, the feather keratin dispersion also yielded more acid-precipitable protein than did the other dispersions, since precipitation of each of the dispersed proteins was almost quantitative. In 0.5 *M* Na₂S, extent of dispersion was nearly the same for the four keratins, but hog hair and wool gave higher yields of acid-precipitable protein than did feathers

and hoof. Hog hair and wool gave higher yields of acid-precipitable protein from 0.5 M Na_2S dispersions than from dispersions in 0.1 M Na_2S . The 0.1 M Na_2S dispersions were allowed to stand at room

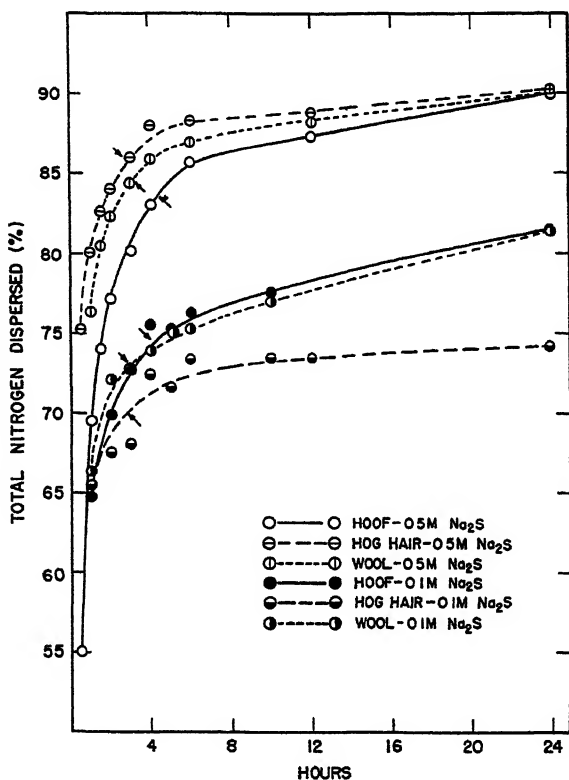


FIG. 4

Rates of Dispersion of Hoof, Hog Hair, and Wool Keratins

7.5 g. keratin per 100 ml. of Na_2S solution at 30°C. Arrows indicate calculated points at which the rate of dispersion had decreased to 1.5 per cent of original keratin per hour.

temperature for a week in stoppered flasks. At the end of this time the hog hair dispersion had set to a gel; the other dispersions remained unchanged in appearance. Upon acidification to pH 4.2, the yield of precipitate from the hog hair dispersion was only slightly less than that from the freshly prepared dispersion; the yield from the feather

dispersion had decreased to 60 per cent of its original value. The hoof and wool dispersions were more stable than that of the feather keratin, but were less stable than the hog hair dispersion.

To determine whether particle size might influence the rate or extent of dispersion of a keratin, 7.5 g. portions of hoof keratin powder, obtained by grinding hoof filings in a Wiley mill equipped with a 20-mesh screen, were digested in 100 ml. portions of 0.1 *M* Na₂S solution at 30°C., and extent of dispersion was determined at hourly intervals. The first five values were, respectively, 72, 75, 78, 79, and 79 per cent dispersion.

TABLE V

Extent of Dispersion and Yield of Acid-Precipitable Fraction in Dispersions of Different Keratins

(7.5 g. keratin per 100 ml. 0.1 *M* and 0.5 *M* Na₂S at 30°C.)

Digestion continued until the rate of dispersion had decreased to 1.5 per cent per hr.

Keratin	Digestion time		Dispersed N	N in Acid-Precipitable Fraction	
	hr.	min.	per cent of total	per cent	per cent
				of total	of dispersed
0.1 M Na ₂ S					
Feather	1	50	82	79	97
Hoof	3	50	73	67	92
Hog hair	2	50	70	70	99
Wool	2	50	69	68	98
0.5 M Na ₂ S					
Feather	2	30	84	69	82
Hoof	4	30	83	69	84
Hog hair	2	50	85	80	94
Wool	3	10	86	78	91

Comparison of these results with those presented in Table V and Fig. 4 shows that the rate of dispersion of hoof keratin is dependent on the degree of comminution of the keratin. The observation that finely ground hoof is dispersed more quickly than hoof filings, with consequent shorter exposure to alkali, indicates that higher yields of acid-precipitable protein might be expected with the use of the ground material.

Throughout the course of this work it was observed that the acid precipitate from each keratin dispersion in a sulfide solution of low molarity (0.1 *M* or less) appeared as a somewhat tough and elastic mass; as the concentration of Na₂S used for dispersion was then increased, the precipitate became more granular. Also as other factors, such as

keratin:sulfide ratio and time, were varied in such a way that theyield of the acid-precipitable fraction was decreased, the precipitate became less coherent. It would thus appear that a correlation exists to some extent between degradation of the keratin and granularity of this product.

SUMMARY

The effects of temperature, time, Na_2S concentration, and ratio of keratin to Na_2S upon the extent of dispersion of feather keratin in Na_2S solutions and upon the yield of material obtained by acidification of such dispersions were studied. The following combination of the above factors was found to yield maximal dispersion of feather keratin with minimal degradation of the dispersed protein: digestion with 0.1 M Na_2S (100 ml. of solution per 7.5 g. of keratin) for about 2 hours at 30°C . The dispersed protein was recovered nearly quantitatively by acidification of the dispersion to pH 4.2. Feather keratin is more readily dispersed and less stable in solutions of Na_2S than are the keratins of hoof, hog hair, and wool.

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The Partial Purification and Some of the Properties of the Enzyme in Rat Liver That Forms Hydrogen Sulfide from Cysteine¹

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INTRODUCTION

A few of the properties of the enzyme in rat liver that produces hydrogen sulfide from cysteine have been previously described (1). Other workers have described the properties of the same enzyme in dog liver in more detail (2, 3). The work reported below deals with a partial purification of the enzyme, the effect of a number of inhibitors upon it and a few of its other properties.

METHODS

The rats used were mature albino rats maintained on a Purina dog chow diet fed *ad libitum*. They were killed by cutting their throats and allowing them to bleed as much as they would. The livers were removed immediately after death and ground in a mortar or a Waring Blendor with twice their weight of physiological saline or Ringer-phosphate solution of pH 7.4. The mixture was then centrifuged and the solid residue discarded. The Ringer-phosphate extracted somewhat more enzyme than did the saline. Toluene was used as a preservative. Such solutions were the starting material.

Hydrogen sulfide and ammonia formation were measured as described previously (1). From Fig. 1 it may be seen that under the conditions used the H_2S formation is a linear function of time of reaction for about two hours. For the comparison of enzyme preparations of different degrees of purity, the H_2S produced per unit of nitrogen has been used.

PURIFICATION

The treatment of the original extracts with chloroform (4) was described previously (1). This step has been retained as a routine pro-

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Contribution No. 491 from the Department of Chemistry, University of Pittsburgh.

cedure. The purification obtained may be seen in Table I. The chloroform treatment could be repeated with some further purification, but the step was not of any great value. After the chloroform treatment the enzyme was precipitated with two volumes of acetone at -5.0°C . The precipitate was washed with ether at -5.0°C . to remove the acetone and then dried *in vacuo*. The dry powder was quite stable,

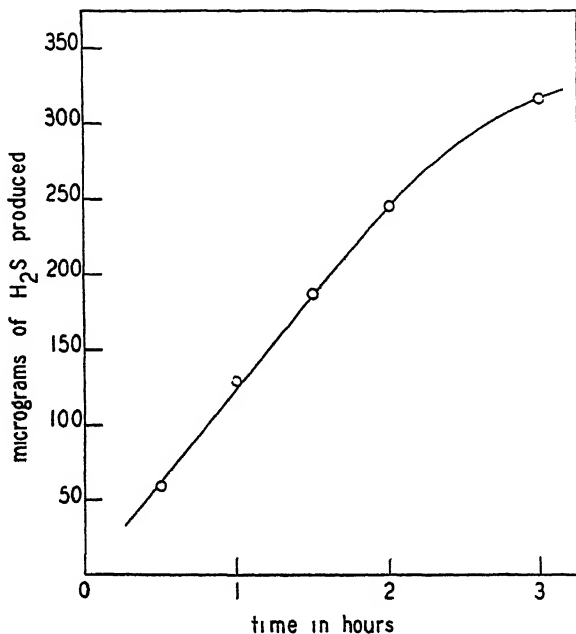


FIG. 1

Rate of Production of H_2S from *l*-Cysteine

The enzyme preparation was a saline solution made from an acetone precipitate (see below). Phosphate buffer of pH 7.4 was added. The cysteine present was 0.2 ml. of 0.1 *M* in a final volume of 2.4 ml.; $T = 37^{\circ}\text{C}$.

e.g., one preparation lost only 22 per cent of its activity in one year. Extraction of the powder with water, saline, or phosphate buffer yielded an active enzyme preparation. The extent of purification and the loss involved in this step may be seen in Table I. The enzyme could be adsorbed on $\text{Ca}_3(\text{PO}_4)_2$ gel, prepared according to Tsuchihashi (5), at pH 7.0. Per ml. of enzyme preparation, 12 mg. of $\text{Ca}_3(\text{PO}_4)_2$ were used. We were not able to elute the enzyme satisfactorily by changing the pH

as Laskowski and Fromageot (3) did, but we did obtain active preparations by using glycerol at pH 8.0. About 80 per cent glycerol was the most effective. Sodium β -glycerophosphate (0.3 *M*) at pH 8.0 was also an effective eluent. Alumina C γ (6) was almost as effective as $\text{Ca}_3(\text{PO}_4)_2$ as an adsorbing agent, and in this case, too, elution was brought about by glycerol and sodium β -glycerophosphate. The extent of purification and the loss for the $\text{Ca}_3(\text{PO}_4)_2$ experiments are again shown in Table I. It may be observed that the activity per mg. of nitrogen has been increased 10 to 15 times by the steps listed. These

TABLE I
Activity and Yield† of Enzyme after Certain Purification Procedures*

Saline extract	CHCl ₃ treated extract		Solution from acetone precipitate		Glycerol eluate from $\text{Ca}_3(\text{PO}_4)_2$	
Activity	Activity	Yield	Activity	Yield	Activity	Yield
33	70	74	112	77		
27	65	89	106	73		
21	69	81	82	55		
28	75	82	109		410	76
			78		348	54
					370	79

* Activity is expressed as micrograms of H_2S produced per mg. of Kjeldahl nitrogen in a 2 hour test at 37°C. with 0.2 ml. of 0.1 *M* *L*-cysteine in a final volume of 2.4 ml. (pH 7.4).

† Yield is expressed as per cent recovery of the activity present before the treatment.

activities are of the same order as the best value reported by Laskowski and Fromageot (3) for their preparations.

The original extracts could be dialyzed quite successfully, although not without some loss, by putting them in cellophane tubes and dialyzing against running tap water at 8–10°C. However, the partially purified extracts were largely inactivated by the same procedure even at 1°C. Thus, the extracts prepared from the acetone precipitate lost at least 75 per cent of their activity on dialyzing over night in a refrigerator against 100 volumes of distilled water. Such extracts could not be reactivated by undialyzed, heated extracts or by the dialyzate after concentration at 50°C. It was found that, if the dialysis was carried out against Ringer's solution, or a similar salt solution, the recovery was much better. Using a salt solution containing 0.0056 *M* KCl,

0.003 *M* CaCl_2 , 0.0014 *M* MgSO_4 , 0.070 *M* NaCl , and 0.0014 *M* phosphate buffer with a final pH of 7.4, a recovery of 84 per cent was obtained. When the dialysis was carried out against distilled water, the above salt solution did not reactivate the enzyme.

Although some enzyme activity is precipitated by ammonium sulfate, we were unable to recover more than small fractions of the total activity from such precipitates under any of the conditions tested. No activity remained in solution upon saturation with this salt, and addition of the supernatant did not increase the activity of the precipitate. No increase in activity resulted from dialysis of any fraction. These results differ rather markedly from those reported by Laskowski and Fromageot (3) for dog liver. The results with sodium sulfate were somewhat more promising, but here also considerable loss in activity resulted.

Purification by electrophoresis, using the Tiselius apparatus (7) was tried. If the enzyme solution was dialyzed against phosphate buffer and the same buffer was then used in the electrode vessels and connections, the enzyme not only lost activity during the dialysis, but underwent an additional large loss during the electrophoresis. Controls treated in the same way but without the passage of current showed no such loss of activity. The current was about 10 milliamperes and the temperature was $+4^\circ\text{C}$. If Ringer's solution, or a salt solution similar to it, was used instead of the phosphate buffer, better results were obtained both on the dialysis and on the electrophoresis, but losses still occurred making purification by this procedure unsatisfactory. Results of these electrophoresis experiments indicated that the enzyme was isoelectric at about pH 5.9. It showed optimum activity at pH 7.1 to 7.4.

INHIBITION STUDIES

Although the properties of the enzyme clearly indicate it to be a protein, we have tested the inactivating effect of trypsin as an additional indication. A 1 per cent suspension of trypsin (Pfanstiehl "1-110") added to the chloroform treated extract at pH 7.3 caused 48 per cent inhibition in one hour and 96 per cent inhibition in 23 hours.

The losses encountered in the $(\text{NH}_4)_2\text{SO}_4$ precipitation suggested that salts as such might affect the activity. A possible example of such effect is the fact that enzyme preparations in veronal buffers show less activity than the same preparation in phosphate buffer of the same pH. To test the question further, a number of salts were added to enzyme

solutions, and the effects found are listed in Table II. It is apparent that the salts differ considerably in their effect. The inhibiting effect of these rather high concentrations of salts should be distinguished from the beneficial effect of small amounts in the dialysis experiments.

TABLE II
Inhibition by Salts

Enzyme preparation	Salt	Enzyme activity* without salt	Activity in presence of salt	Inhibition
		(γH_2S /ml.)	(γH_2S /ml.)	per cent
CHCl ₃ -treated extract	0.42 M CH ₃ ·CO·OK	154	141	8
CHCl ₃ -treated extract	0.42 M CH ₃ ·CO·ONa	155	138	11
CHCl ₃ -treated extract	0.42 M CH ₃ ·CO·ONH ₄	252	124	51
CHCl ₃ -treated extract	0.42 M NaCl	155	100	35
CHCl ₃ -treated extract	0.42 M KCl	154	89	42
Solution from acetone precipitate	0.42 M NH ₄ Cl	173	65	63
CHCl ₃ -treated extract	0.42 M Na ₂ SO ₄	252	179	29
CHCl ₃ -treated extract	0.42 M (NH ₄) ₂ SO ₄	252	94	62
Solution from acetone precipitate	0.42 M MgSO ₄ †	89	13	86
Solution from acetone precipitate	0.42 M NaNO ₂	140	31	78
CHCl ₃ -treated extract	0.42 M NaNO ₃	154	22	86
Solution from acetone precipitate	0.42 M NH ₄ NO ₃	124	4	97
CHCl ₃ -treated extract	0.42 M KNO ₃	155	0	100
Solution from acetone precipitate	0.01 M KNO ₃	164	160	2

* The tests were run for 2 hours at 37° C. in phosphate buffer of pH 7.4, and 0.2 ml. of 0.1 M l-cysteine was added as substrate.

† Bicarbonate buffer rather than phosphate.

It was previously reported that KCN inhibited the enzyme (1). This result suggested that a heavy metal compound might be involved, so other compounds that are known to inhibit some reactions catalyzed by heavy metal complexes were tested. As shown in Table III the materials tested did not show any marked inhibition even at the rela-

tively high concentrations listed. In the carbon monoxide experiment the atmosphere was practically 100 per cent carbon monoxide.

It was found that the addition of arsenous oxide to the buffered solution caused a rather strong inhibition. So far as we are aware, the chemistry of arsenite inhibition of enzymes (8) is not known, but at least some of the reactions inhibited seem to be concerned with keto acids (9). Since the KCN inhibition might also be due to reaction with a carbonyl group, other carbonyl reagents were tested. As Table IV shows, they exerted a very strong inhibiting effect on both H_2S and

TABLE III
Effect of Compounds that Inhibit Metal-Complex Catalysts

Inhibitor	Enzyme activity*	Activity in presence of inhibitor	Inhibition
	($\gamma H_2S/ml.$)	($\gamma H_2S/ml.$)	per cent
0.01 M Potassium ethyl xanthate	214	164†	23†
0.01 M Sodium azide	199	159	20
0.01 M Sodium diethyldithiocarbamate	121	98	18
Carbon monoxide atmosphere	229	193	16
0.01 M Thiourea	199	183	8
0.001 M 8-Hydroxyquinoline	231	222	4
0.01 M Sodium pyrophosphate	242	246	0

* The enzyme preparation used was a solution prepared from an acetone precipitate. The cysteine concentration was 0.0083 M. The experiments were run for 2 hours at 37°C. and pH 7.4.

† 20 micrograms of H_2S were obtained from the same concentration of potassium ethyl xanthate in the absence of enzyme, and the figures given have been corrected for this amount. None of the other inhibitors yielded any H_2S in this way.

NH_3 formation. Attempts to reverse the phenylhydrazine inhibition with benzaldehyde were not successful.

Compounds with a structure similar to cysteine might be expected to inhibit the reaction. As Table V shows, the amino acids, alanine, and methionine, did not show any inhibition. However, the thiol compounds: sodium thioglycolate, glutathione, *d*-cysteine², and the two

² The *d*-cystine was kindly furnished by Professor V. du Vigneaud. Samples of the two forms of α -amino- β -thiol butyric acid were furnished by Professor H. E. Carter and by Professor du Vigneaud. The enzyme did not produce H_2S from these compounds alone.

forms (A and B) (10) of α -amino- β -thiol butyric acid² were inhibitors. In the case of sodium thioglycolate, the evidence indicates that the

TABLE IV
Inhibiting Effect of Various Reagents

Enzyme Activity* (control)	Inhibitor	Molar concentration of inhibitor	Inhibition of H ₂ S	Inhibition of NH ₄ †
(γ H ₂ S ml)			<i>per cent</i>	<i>per cent</i>
207	KCN	2.5×10^{-2}	82	72
		1.0×10^{-2}	65	61
		5.0×10^{-3}	54	45
		2.5×10^{-3}	41	
151	As ₂ O ₃	1.0×10^{-2}	100	97
		5.0×10^{-3}	100	90
		2.5×10^{-3}	97	80
		1.0×10^{-4}	54	53
144	Phenylhydrazine	1.0×10^{-2}	100	100
		5.0×10^{-3}	100	100
		2.5×10^{-3}	100	92
		5.0×10^{-4}	93	96
		1.0×10^{-4}	53	
138	Semicarbazide	1.0×10^{-2}	100	100
		1.0×10^{-3}	90	91
		1.0×10^{-4}	61	82
124	Hydroxylamine	1.0×10^{-3}	100	
		5.0×10^{-4}	100	100
		1.0×10^{-4}	96	100
157	NaHSO ₃	1.0×10^{-2}	70	75

* The enzyme solution used was made from an acetone precipitate. The cysteine concentration was 8.3×10^{-2} M. The experiment was run for 2 hours at 37°C. and pH 7.4

† Under the conditions employed, controls produced 1 mole of NH₄ per mole of H₂S.

inhibition was of the competitive type (Fig. 2) although the intercept was not quite the same for the two curves (11).

Of all the compounds tested, glycerol was the only one that showed

TABLE V
Effect of Amino and Thiol Compounds

Substrate	Inhibitor	Enzyme activity without inhibitor	Activity in presence of inhibitor	Inhibition
		(γH_2S ml.)	(γH_2S ml.)	per cent
0.0125 M L-Cysteine	0.0139 M DL-Alanine	185	187	0
0.0143 M L-Cysteine	0.0178 M DL-Methionine	175	178	0
0.0053 M L-Cysteine	0.0083 M Sodium thioglycolate	133	95	27
0.0125 M L-Cysteine	0.0125 M Glutathione	178	112	37
0.0083 M L-Cysteine	0.0083 M D-Cysteine	243	208	14
0.0080 M L-Cysteine	0.0080 M α -Amino- β -thiol butyric acid A	166	56	66
0.0080 M L-Cysteine	0.0080 M α -Amino- β -thiol butyric acid B	166	47	71

The enzyme preparation used in the first five experiments was a solution prepared from an acetone precipitate; in the last two experiments a chloroform-treated extract was used. The experiments were run for 2 hours at 37°C. and pH 7.4.

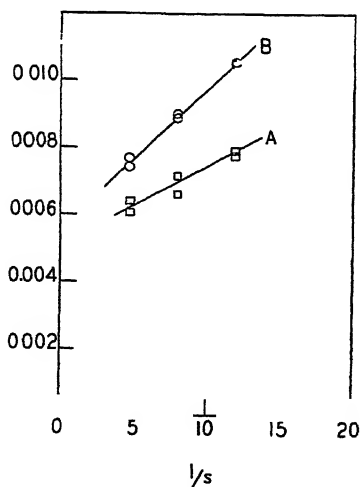


FIG. 2

Competitive Nature of Inhibition by Sodium Thioglycolate Shown by the Method of Lineweaver and Burk (11)

Curve A: No inhibitor present. Curve B: 0.0083 M sodium thioglycolate present. S is the concentration of L-cysteine in mols per liter. V is the reaction velocity in micrograms of H_2S produced per hour. The enzyme solution was an aqueous solution prepared from an acetone precipitate and buffered at pH 7.4 with phosphate.

any accelerating effect. Since a rather high concentration (2.0 *M*) was required to produce an activation (30 per cent), the effect may be due to change in solvent.

The sensitivity of the enzyme to dialysis, electrophoresis, and salt concentration suggested that it might be a metal compound. In order to test what metals were present, emission spectra were run on several enzyme preparations of different degrees of purity. The results showed that iron, copper, magnesium, and sodium were present in appreciable amounts in all the preparations tested. Although no precise quantitative measurements were made, the plates suggested that the concentration of magnesium varied less with purification of the enzyme than did that of the others. This finding in conjunction with the rest of the data suggests that if any one of the metals detected is a component part of the enzyme, it is magnesium. However, the present data does not rule out the possibility that all the metals detected are impurities.

DISCUSSION

Some of the evidence presented above indicates that a carbonyl group may be concerned in the reaction under discussion. If only the formation of H_2S is considered, it is possible that the carbonyl group may arise from the cysteine. Thus, the first step might be an oxidative deamination resulting in the formation of thiol pyruvic acid. Reaction of the inhibiting reagents with the carbonyl group might prevent the formation of hydrogen sulfide. However, the evidence indicates that the inhibiting agents also prevent the formation of ammonia (Table IV) which would seem to rule out such a mechanism. It would, therefore, seem probable that the postulated carbonyl group is part of the enzyme and is necessary for the reaction. There are two obvious ways in which cysteine might react with such an enzyme carbonyl group to form a labile enzyme-substrate complex, i.e., through the amino group or through the thiol group. The fact that alanine and methionine do not inhibit the reaction suggests that the postulated combination does not occur *via* the amino group, while the fact that the thiol compounds do inhibit the reaction, and in at least one case in a competitive manner, suggests that a combination does occur *via* the thiol group. That the addition of the thiol group and the enzyme is not sufficient for completion of the reaction is evident from the fact that the other thiol compounds mentioned do not serve as substrates for the formation of hydrogen sulfide.

SUMMARY

The enzyme in rat liver that produces H_2S from cysteine has been partially purified and some of its properties studied. Potassium cyanide, arsenous oxide, and certain carbonyl reagents were found to be strong inhibitors of the reaction. Other compounds that are known to inhibit heavy metal catalyzed reactions did not cause appreciable inhibition. The presence of small concentrations of certain salts was necessary to prevent inactivation during dialysis or electrophoresis, but greater concentrations of salts strongly inhibited the reaction. Amino acids did not inhibit the reaction, but thiol compounds did. The inhibition by sodium thioglycolate was shown to be competitive in nature.

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Synthesis of Pyridoxin by a "Pyridoxinless" X-ray Mutant of *Neurospora sitophila*

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INTRODUCTION

In a new approach to the study of the relation of genes to biochemical reactions, Beadle and Tatum (1) recently obtained x-ray induced mutants of the mold *Neurospora*, which are unable to synthesize pyridoxin (*N. sitophila*), the thiazole portion of thiamine (*N. sitophila*), and *p*-aminobenzoic acid (*N. crassa*), respectively. The normal strains from which the mutants were derived can synthesize these particular vitamins since growth occurs in the absence of these substances, whereas the mutant strains require them for growth. Genetic analyses indicated that the inability of *Neurospora* to synthesize a particular vitamin is associated with the mutation of a single gene. It was suggested that the mutants could be used for the quantitative assay of the vitamins whose synthesis the mutants are no longer able to carry out, and preliminary studies were made on the quantitative response to pyridoxin (vitamin B₆) by the "pyridoxinless" mutant of *N. sitophila*.

While developing a quantitative method for the assay of pyridoxin by use of the above mutant, it was unexpectedly discovered that this pyridoxinless strain can grow normally *without pyridoxin* under sharply defined conditions of pH and nitrogen nutrition.

METHODS

A synthetic medium was used which differed somewhat from that of Beadle and Tatum as shown in Table I.

Ammonium nitrate is omitted since it does not effect growth; ammonium tartrate supplies sufficient available nitrogen. Glucose appears to permit somewhat more abundant growth than sucrose. A trace of zinc is stimulatory.

The medium was distributed in 10 ml. amounts in 50 ml. Erlenmeyer flasks. Glucose and pyridoxin were each sterilized separately and added to the auto-

claved medium as required. The medium was buffered at different pH levels with phosphate buffer (Sorensen's) to a final concentration of $M/15$. When phosphate buffer was used, KH_2PO_4 was omitted from the medium.

Hydrogen ion concentration was measured with a glass electrode with an accuracy of 0.1 pH unit. Initial pH values of the media were determined after sterilization since autoclaving caused a pH drop of 0.2 to 0.4 of a unit.

Stock cultures of *Neurospora*¹ were grown on Sabouraud agar slants and after sporulation were stored in the refrigerator until used. Inoculum was prepared by carefully removing a small portion of growth, consisting primarily of conidia, and making a uniform spore suspension in 10 ml. of sterile water. Each 10 ml. of medium received one drop of the spore suspension delivered from 1 ml. pipette.

Cultures were incubated for 5 days at 30°C. and then steamed for 5 minutes to kill the spores. The fungus material was removed and pressed dry between paper

TABLE I
Composition of Synthetic Media for the Growth of Neurospora

Ingredients	Medium of Beadle and Tatum	Modified medium
NH_4 tartrate.	5.0 g.	5.0 g.
NH_4NO_3	1.0 g.	—
KH_2PO_4	1.0 g.	1.0 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.	0.5 g.
NaCl.....	0.1 g.	0.1 g.
$CaCl_2$	0.1 g.	0.1 g.
$FeCl_3$	5.0 mg.	5.0 mg.
$ZnSO_4 \cdot 7H_2O$	—	1.0 mg.
Biotin.....	4.0 γ	4.0 γ
Glucose.....	—	15.0 g.
Sucrose.....	10.0 g.	—
H_2O distilled.....	1 liter	1 liter

towels and finally dried at 80°C. overnight before being weighed. All results are the averages of duplicate or triplicate treatments. Modifications in the procedure are described in the text as introduced.

EXPERIMENTS

The reaction of the medium is approximately pH 5. The buffering effect of the tartrate prevents the pH of the medium from falling below approximately 3.5 during growth of the fungus. The normal response to pyridoxin by the pyridoxinless mutant of *N. sitophila* is shown in Fig. 1.

¹ We are greatly indebted to Dr. G. W. Beadle for the *Neurospora* cultures used in this investigation.

Growth is proportional to the pyridoxin content of the medium, and 1 γ of pyridoxin yields 40 to 50 mg. of cell substance.

Effect of pH

To determine the influence of higher pH, 0.5 and 1.0 per cent sodium acetate were added, and ammonium tartrate was replaced by ammonium nitrate (2 g./liter). Unexpectedly, maximum growth occurred in the control flasks which had not received pyridoxin. The initial pH values

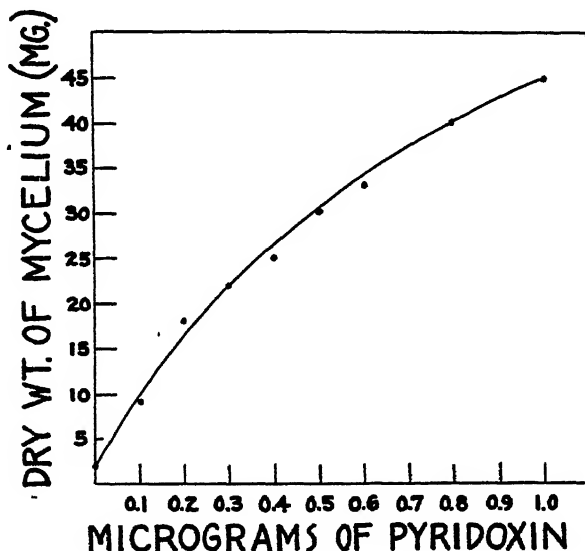


FIG. 1

Response of *N. sitophila* to Pyridoxin

of the different media were not recorded, but the values after growth were pH 5.5 and pH 7.9 with 0.5 and 1.0 per cent acetate as compared to pH 3.5 in the standard medium containing 2 γ of pyridoxin. These results pointed to a specific effect of hydrogen ion concentration on the growth of the pyridoxinless mutant. The fungus was therefore grown in the standard medium adjusted to various pH levels with phosphate buffer, with and without pyridoxin.

The results (Table II) clearly indicate that hydrogen ion concentration has a critical influence on the growth of the mutant in conjunction with its pyridoxin requirements. Without pyridoxin, the fungus does not

develop in the range of pH 4.9 through pH 5.4. At pH 5.6, a very small amount of growth appears, increasing to about half-maximum growth at pH 5.8, almost maximum at pH 6.0, and maximum growth (36 to 41 mg.) in the range pH 6.2 to pH 7.3, inclusive. pH 7.8 is unfavorable for development of the fungus, since growth does not occur even when pyridoxin is present. As expected, in the presence of pyridoxin the mutant grows normally at all pH levels except at pH 7.8 which is inhibitory. The amount of cell material built up without added pyridoxin at pH 6.0 to pH 7.3, inclusive, is similar to that formed in the presence

TABLE II

Effect of pH on the Pyridoxinless Mutant of N. sitophila in the Presence and Absence of Pyridoxin

Initial pH	Without Pyridoxin		1 γ Pyridoxin Added	
	Final pH	Dry wt. of mycelium mg.	Final pH	Dry wt. of mycelium mg.
4.9	—	0	4.1	24
5.0	—	0	3.8	34
5.2	—	0	4.0	33
5.4	—	0	4.0	32
5.6	5.4	trace	4.3	35
5.8	4.5	17	4.3	37
6.0	4.6	29	4.7	38
6.2	5.0	36	5.2	36
6.5	5.6	36	5.9	33
6.6	6.0	37	6.0	28
7.3	6.4	41	6.4	38
7.8	7.7	0	7.6	0

of the vitamin. Thus, the requirement for pyridoxin by the mutant strain can be eliminated by simply adjusting the pH of the medium.

In cultures grown at initial pH 5.8 to pH 6.5 without pyridoxin, the final pH values ultimately fall as a result of growth below the lowest pH at which the mutant can initiate growth. An experiment was set up to establish whether the critical effect of pH applies at all stages of development or is limited to the initiation of growth.

A series of 125 ml. Erlenmeyer flasks containing 30 ml. of medium adjusted to pH 6.7 with phosphate was inoculated with conidia of the pyridoxinless mutant. After 1, 2, 3, and 4 days of growth, respectively, three cultures were harvested, and three additional cultures were adjusted to pH 4.8 with sterile 2 N HCl and replaced in the incubator.

After five days, all remaining cultures, including three which had not been adjusted, were harvested.

Table III shows that the weight of mycelium in the cultures adjusted after 1, 2, 3, and 4 days is significantly less than the untreated cultures and is proportional to the length of the growth period prior to the pH change. This means that normal growth in the absence of pyridoxin proceeds only if the pH is maintained above 5.8 at all stages. Lowering the pH below this at any stage stops or greatly retards growth.

A small amount of growth occurs after the hydrogen ion concentration is brought below the critical pH. This may be due to utilization of

TABLE III
Effect of pH Adjustment at Different Stages of Growth

Replicates	Dry weight of mycelium								Unad-justed, 5 days
	Adjusted to pH 4.8 after								
	1 day		2 days		3 days		4 days		
	Har-vested	Incub-ated 5 days	Har-vested	Incub-ated 5 days	Har-vested	Incub-ated 5 days	Har-vested	Incub-ated 5 days	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	1	7	21	48	83	81	151	174	177
2	2	7	21	51	83	84	155	171	179
3	1	6	24	47	95	105	154	—	183
Average..	1	7	22	49	87	90	153	173	180

stored pyridoxin (see below) or to the ability of the cells to resist rapid changes in pH. The cultures acidified after 4 days continued to grow for an additional day since their pellicles weighed only slightly less than those of the 5 day old control cultures. For maximum growth in the absence of pyridoxin the pH must remain above the critical range during the whole growth period.

The possibility of the presence of contaminating microorganisms developing only above pH 5.8 and synthesizing pyridoxin, which *Neurospora* then utilizes for growth, is ruled out since repeated microscopic examinations failed to reveal any trace of contamination. Nor is it likely that the phenomenon is dependent upon traces of pyridoxin carried over from Sabouraud's medium in the inoculum since full growth

has been obtained in all of six serial subcultures in media at pH 6.5. Also, the modifications made in the medium of Beadle and Tatum are not critical because equivalent results are obtained in that identical medium (see Table I).

As previously indicated, the mutant develops normally at pH 6 to 7 without pyridoxin when glucose or sucrose is the source of carbon and energy. Similar results are obtained with maltose, raffinose, and xylose, although less growth occurs with raffinose than with the other carbohydrates. The fungus is unable to utilize lactose, mannitol, or inulin. Growth without pyridoxin therefore appears to be independent of the

TABLE IV

Influence of Nitrogen Source, pH and Pyridoxin on Growth of the Pyridoxinless Mutant of Neurospora sitophila

Nitrogen Source*	pH 4 to 5		pH 6 to 7	
	Without pyri-doxin	1 γ pyri-doxin	Without pyri-doxin	1 γ pyri-doxin
Nitrate-nitrogen: NaNO_3 , KNO_3 , $\text{Ca}(\text{NO}_3)_2$	—	+	—	+
Nitrite-nitrogen: NaNO_2	—	+	—	+
Ammonium-nitrogen: NH_4NO_3 , NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$, NH_4 tartrate	—	+	+	+
Amino-nitrogen: d-glutamic acid, glycine, dl-leucine, l-tryptophan, l-proline	—	+	—	+
Amide-nitrogen: asparagine, acetamide	—	+	—	+
Other forms of nitrogen: adenine, egg albumen	—	+	—	+

* 0.2 per cent concn. used with exception of 0.5 per cent asparagine.

+ = good growth, — = no growth.

type of available carbohydrate supplied to the mutant. However, this is not the case with the nitrogen source (Table IV).

At pH 4 to 5, growth of the mutant does not take place with any of the nitrogen compounds unless pyridoxin is present. At pH 6 to 7, the fungus develops without pyridoxin *only with ammonium-nitrogen*: nitrate, nitrite, amino, amide, and other forms of nitrogen require the presence of pyridoxin irrespective of the type of compound employed whereas all forms of ammonium-nitrogen permit growth without pyridoxin.

The tentative conclusion may be made that the x-ray induced gene mutation in *N. sitophila* has not destroyed the ability of the fungus to

synthesize pyridoxin but has limited the conditions under which such synthesis can take place. This is only at a pH of 5.8 or greater with ammonium compounds as sources of nitrogen. The normal parent strain grows independently of pH and nitrogen source. The possibility exists that growth, in the absence of pyridoxin, may proceed without involving pyridoxin, and it may not be necessary for the fungus to synthesize the vitamin. This possibility seems unlikely in view of the numerous published experiments on the growth factor requirements of microorganisms which show that growth in the absence of a particular

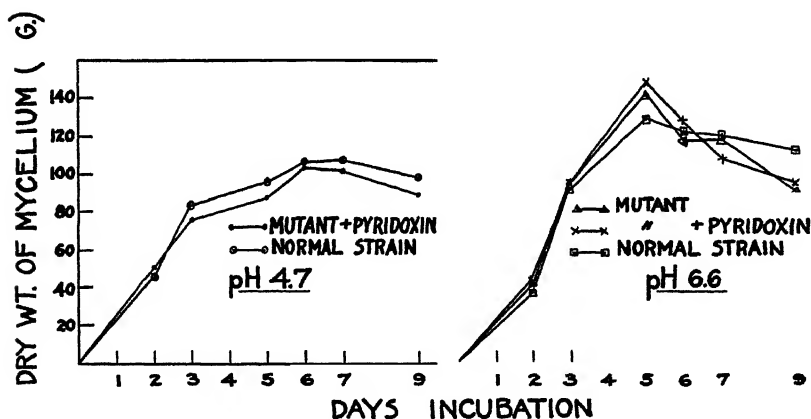


FIG. 2

Growth Curves of Normal and Mutant Strains of *Neurospora* at pH 4.7 and pH 6.6

vitamin is generally associated with the ability of the organism to synthesize it (2, 3, 4, 5).

The presence of pyridoxin was demonstrated in cultures of the mutant and of its normal parent strain, the latter grown without the vitamin. Both strains were cultured at pH 4.8 and pH 6.2 in quadruplicate in 100 ml. of stock medium in 500 ml. Erlenmeyer flasks. To obtain growth of the mutant at the lower pH, 10 γ of pyridoxin was added to each flask. After incubation for 5 days, dry weights were obtained from two cultures of each treatment. Each of the remaining pellicles was pressed dry between paper towels, cut into small pieces, and autoclaved with 15 ml. of 1 *N* HCl at 15 lbs. pressure for one hour to extract pyridoxin. The extracts were filtered through paper, adjusted to pH 5, diluted with water to 25 ml., and sterilized by autoclaving. The pyridoxin content of each

fungus extract was determined by the growth response of the *Neurospora* mutant in media at pH 4.9 with different levels of extract added and also by means of the *Lactobacillus casei* assay method (6). The results are presented in Table V.

The mycelium of the normal strain grown at pH 4.8 contained as much pyridoxin as the mutant grown at the same pH with pyridoxin. More significant, however, is the finding that cell material of the mutant and of the normal strain cultured at pH 6.2 without pyridoxin contained essentially the same amount of that vitamin. The absolute values are not strictly quantitative for two reasons listed below, but the comparative values show that pyridoxin is synthesized by the mutant at pH 6.2 in amounts equivalent to those by the normal strain. The mutant is stimulated by thiamin when grown at pH 4 to 5 with submaximum

TABLE V

Pyridoxin Content of Cell Material of the Normal and Mutant Strains of N. sitophila

Cultures and treatment	Total cell material mg.	Pyridoxin per 100 mg. mycelium as determined with	
		<i>Neurospora</i> micrograms	<i>L. casei</i> micrograms
pH 4.8			
Mutant + pyridoxin	254	0.91	30.2
Normal strain (no pyridoxin)	269	1.37	47.4
pH 6.2			
Mutant (no pyridoxin)	395	1.01	45.5
Normal strain (no pyridoxin)	306	1.30	

levels of pyridoxin, and the *Neurospora* pyridoxin assay values may therefore in part reflect the stimulatory action of thiamin in the pellicle extracts. Fig. 3 shows that growth of the mutant strain with 0.1 γ of pyridoxin plus 0.5 γ of thiamin is equal to that obtained with 0.4 γ of pyridoxin alone and greater than the latter when 0.6 γ to 3.0 γ of thiamin is present. In the absence of added pyridoxin, thiamin has a small but definite stimulatory action. Thiamin, therefore, can largely, although not completely, replace pyridoxin in the nutrition of the mutant strain. The high values obtained with *L. casei* are probably due in part, at least, to the presence of pseudopyridoxin (7, 8). The comparisons are supported by Fig. 2 which contains growth curves of both strains cultured at pH 4.7 and pH 6.6. This experiment was done with 30 ml. medium in 125 ml. Erlenmeyer flask. When the mutant is supplied with pyridoxin, it develops at the same rate as the normal parent strain

at the lower pH. It appears that the rate of pyridoxin synthesis is not the limiting factor in the growth of the parent. At the higher pH, the rate and total amount of growth is the same for the two strains regardless of the presence or absence of pyridoxin for the mutant. It follows that the rate of synthesis of pyridoxin by the mutant equals that of the parent strain.

Additional experiments have shown that: (a) biotin must be supplied for growth of the mutant and normal strain irrespective of pH or type of nitrogen nutrition, (b) variation in the ammonium tartrate content

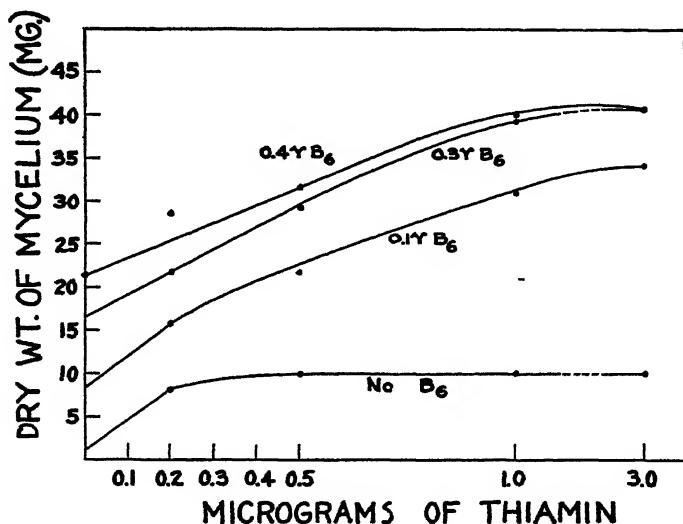


FIG. 3

Growth Stimulation of *N. sitophila* by Thiamin at Submaximum Levels of Pyridoxin

of the medium between 0.1 and 1.0 per cent at pH 6 to 7 without pyridoxin affects only the degree of growth of the mutant, and (c) the pH-ammonium-nitrogen phenomenon is specific for the pyridoxinless mutant of *N. sitophila* since the thiamin and *p*-aminobenzoic acid mutant strains of *N. crassa* require their respective vitamins for growth at pH 6 to 7 with ammonium-nitrogen as well as at pH 4 to 5.

DISCUSSION

As Beadle and Tatum have indicated, the biosynthesis of pyridoxin undoubtedly involves a series of reactions only one of which, presumably,

is blocked by the x-ray induced mutation in *N. sitophila*. These investigators were able to obtain growth of the mutant by supplying it with pyridoxin. Results reported here demonstrate that the genetically blocked reaction can also be "by-passed" by the combined procedures of adjusting the culture medium to pH of 6 or higher and supplying only ammonium compounds to meet the nitrogen demands of the fungus. Under such cultural conditions the mutant develops normally, and its growth is due to a reappearance of the ability to synthesize pyridoxin.

The conditions of pH and nitrogen nutrition controlling the synthesis of pyridoxin by the mutant are highly specific. This receives support from the finding that the thiamin and *p*-aminobenzoic acid requiring mutants of *Neurospora* are not influenced by these two conditions. The limited data available suggest that these physiological requirements of the mutant for synthesis of pyridoxin reflect the specific biochemical changes produced by the x-ray irradiation, *i.e.*, an alteration in nitrogen metabolism involved in the formation of pyridoxin, this alteration being a function of pH. It seems possible that an additional tool is now available—that of determining the physiological conditions necessary to "by-pass" a particular mutational effect—which can be used to analyze more closely the biochemical aspects of gene mutations.

SUMMARY

A pyridoxin-requiring mutant of *N. sitophila* grew normally in the absence of the pyridoxin if the culture medium was buffered with sodium acetate. On adjusting the medium to different pH levels, appreciable growth of the fungus occurred only at pH 5.8 or higher. At these pH values it is also necessary to supply the mutant strain with ammonium compounds as nitrogen sources, other forms of nitrogen being unsuitable. Under these conditions, the ability to synthesize pyridoxin is restored. Other aspects of the pH-ammonium-nitrogen relationship to pyridoxin synthesis in the mutant and also some genetic implications are discussed.

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A Rotatory Dispersion Study of α -Amino Acids

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INTRODUCTION

The purpose of this investigation was to observe the rotatory dispersion in the visible region of the spectrum of selected α -amino acids as well as the sodium and hydrochloride salts; to analyze the observed rotatory dispersion for the sign of partial rotation of the amino and carboxyl groups; and to indicate from the sign of rotation of these groups in α -amino acids of known configuration a method of assigning a configuration to a new α -amino acid.

Chemical and physical methods have both been used in assigning a relative configuration to α -amino acids. Thus, Fischer and Raske (1), and Karrer (2) related the amino acids by chemical means when they converted one compound to another by reactions not involving the asymmetric center. Barrow and Ferguson (3) used another chemical method when they related the configuration of *l*(+)-alanine and *l*(+)-valine by converting *l*(+)-alanine to (-)- α -methylisobutylamine and *l*(+)-valine to (+)- α -methylisobutylamine with reactions not involving the asymmetric center. The method used by Clough (4) and Freudenberg (5), in which compounds were considered to be configurationally related when corresponding derivatives showed the same relative change in molecular rotation, was based on physical data. Lutz and Jirgenson (6) used a similar physical method when they showed that the rotation of configurationally related α -amino acids changed in an analogous manner with change in pH.

Inasmuch as the sign of the partial rotation of a group attached to the asymmetric center was found to be the same for configurationally related compounds in an homologous series, the determination of the sign of the partial rotation of the amino and the carboxyl groups of α -amino acids affords a physical method of determining their configuration. This method was used extensively by Levene and Rothen (7) for compounds other than amino acids and developed from the suggestion of Tchugaeff (8) that the optical rotation of a molecule was equal to the sum of the partial rotations of the groups attached to the asymmetric center. Although, rotatory dispersion measurements have been made for tyrosine (9, 10), aspartic acid (11), glutamic acid (11, 10), and leucine (10) no comparison of the

TABLE I
The Specific Rotations of the α -Amino Acids

Amino Acid	Weight of Sample (g.)	Length of Tube (dm.)	Equiva- lents of Acid or Base	Source	Wave length (m μ)								
					440	460	480	500	540	580	620	660	Na _D
<i>L</i> - α -Alanine at 22°C. in 25 cc. solution	1.00	2	0	a	8.40	6.02	4.66	3.74	2.71	2.04	1.61	1.36	1.96
	.4210	2	1-HCl	a	36.5	30.5	22.9	19.1	14.3	11.4	8.57	6.35	10.2
	.710	2	1-NaOH	a	—	6.23	5.66	4.72	3.78	3.21	2.64	2.27	2.93
	.1100	2	3-HCl	a	39.3	34.6	27.5	23.3	18.3	15.1	12.1	10.3	14.3
<i>d</i> -Valine at 25°C. in 25 cc. solution	.674	2	3-NaOH	a	16.8	12.3	9.90	7.92	6.34	4.95	3.96	3.17	4.65
	.8953	2	0	b	-12.7	-10.6	-9.93	-8.38	-7.26	-5.60	-4.89	-4.33	-5.56
	.7933	2	1-HCl	b	-49.5	-43.0	-37.7	-34.7	-28.2	-24.0	-21.3	-17.7	-23.6
	.8900	2	1-NaOH	b	-30.4	-27.8	-24.3	-22.8	-19.0	-16.2	-14.1	-12.5	-15.7
<i>L</i> -Leucine at 25°C. in 25 cc. solution	.3253	4	0	c	-15.1	-14.3	-13.9	-13.5	-12.9	-11.8	-10.8	-10.0	-11.5
	.8279	2	1-HCl	c	24.5	21.8	18.1	16.3	13.0	10.6	9.06	7.56	10.3
	.8011	2	1-NaOH	c	21.1	17.5	14.7	12.5	10.0	7.80	6.40	5.46	7.52
	.3201	4	0	b	-53.0	-50.8	-49.2	-45.6	-39.3	-33.8	-29.5	-26.5	-33.4
<i>L</i> -Phenylalanine at 25°C. in 25 cc. solu- tion	.7923	2	1-HCl	b	—	-15.0	-14.4	-13.9	-13.0	-11.8	-11.0	-10.0	-11.6
	.8559	2	1-NaOH	b	—	3.66	2.19	1.17	—	-1.17	-1.90	-2.34	-1.17

L-Tyrosine at 25°C. in 25 cc. solution	.4769 .9554	2 2	2-HCl 2-NaOH	c c	— —	— —	-16.5 -20.7	-15.7 -19.0	-14.2 -16.8	-12.9 -14.7	-11.8 -13.1	-10.8 -11.6	-12.6 -14.4
L-Ilistidine* at 28°C. in 25 cc. solution	.8264 .8727 .9610	2 2 2	0 1-HCl 1-NaOH	d d d	-70.8 8.75 —	-64.4 6.42 -16.4	-62.7 4.85 -15.1	-56.0 3.31 -14.2	-47.6 1.95 -12.2	-41.4 .973 -10.7	-36.1 .584 -9.30	-33.0 .195 -8.25	-39.6 .795 -10.3
L-Proline at 30°C. in 25 cc. solution	.4025 .4309 .4552	2 2 2	0 1-HCl 1-NaOH	d d d	-164 -105 -105	-147 -101 -177	-133 -95.2 -156	-123 -82.6 -143	-103 -69.6 -121	-88.7 -60.0 -103	-77.5 -51.8 -88.2	-68.5 -47.3 -76.2	-86.8 -58.0 -99.1
L-Tryptophan at 31°C. in 25 cc. solution	.2335 .7837 .9261	2 2 2	0 1-HCl 1-NaOH	d d d	-56.3 8.76 —	-49.8 4.78 16.9	-47.2 2.38 10.2	-44.0 .80 7.44	-40.2 -.48 4.34	-35.8 -1.75 3.11	-31.6 -2.71 2.30	-28.9 -3.50 1.90	-34.5 -1.75 2.90
L-Aspartic acid at 27°C. in 25 cc. solu- tion	.1977 .4786 .4012 .4009	2 2 2 2	0 1-HCl 1-NaOH 2-NaOH	d d d d	— — -30.5 —	— 36.0 -29.3 —	— 32.8 -26.5 —	6.65 28.9 -25.2 -5.31	5.70 23.9 -22.4 -4.68	4.76 20.5 -20.3 -4.21	4.12 17.8 -18.1 -3.90	3.46 15.3 -16.2 -3.58	4.60 19.7 -20.0 -4.06
L-Glutamic acid at 24°C. in 25 cc. solution	.5009 .5013 .5010 .5009	2 2 2 2	0 1-HCl 1-NaOH 2-NaOH	c c c c	— 53.8 — —	21.7 51.5 -9.25 22.0	18.7 45.7 — 18.2	16.9 41.9 -8.50 16.0	14.4 33.9 -7.75 12.2	12.4 28.6 -7.00 9.74	10.7 24.9 -6.50 7.91	9.20 21.4 -6.00 6.74	12.1 27.9 -6.95 9.32
L-Lysine† at 28°C. in in 25 cc. solution	.5024 .5020 .5012 .5000	2 2 2 2	0 1-HCl 1-NaOH 2-HCl	d d d d	— — — —	— — — —	22.1 21.5 20.9 38.6	19.9 19.0 18.7 35.7	15.9 15.5 15.0 26.9	13.1 12.7 11.9 23.6	11.2 10.9 10.3 19.1	9.65 9.32 8.75 16.3	12.8 12.3 11.7 22.0

TABLE I—*Concluded*

Amino Acid	Weight of Sample (g.)	Length of Tube (dm.)	Equiva- lents of Acid or Base	Source	Wave length (m μ)								
					440	460	480	500	540	580	620	660	Na ₂
L-Arginine† at 28°C. in 25 cc. solution	.5139	2	0	c	27.4	23.6	20.4	19.0	15.1	12.4	10.6	9.15	12.1
	.5108	2	1-HCl	c	—	25.4	22.8	20.8	16.4	13.6	11.3	9.84	13.1
	.5220	2	1-NaOH	c	—	21.7	20.0	18.8	14.2	11.9	10.2	8.7	11.5
	.4975	2	2-HCl	c	46.2	41.9	36.6	33.3	27.3	22.6	19.2	16.8	22.0
L-Cystine at 31°C. in 25 cc. solution	.1147	2	2-NaOH	d	—	166	144	131	113	95.2	82.0	72.0	92.0
	.1210	2	4-HCl	d	443	418	377	344	290	248	214	191	242
L-Methionine at 28°C. in 25 cc. solution	.7553	2	0	e	—	10.1	9.60	9.26	8.44	7.77	7.12	6.62	7.60
	.8610	2	1-HCl	e	—	35.7	31.9	28.6	23.8	19.7	17.0	14.9	19.3
	.7958	2	1-NaOH	e	9.9	7.4	5.35	4.40	3.13	2.36	1.80	1.57	2.25

* Weighed as L-histidine monohydrochloride monohydrate, but calculated on the basis of the free acid.

† Weighed as L-lysine monohydrochloride, but calculated on the basis of the free acid.

‡ Weighed as L-arginine monohydrochloride, but calculated on the basis of the free acid.

Sources:

a—Resolved; see Facsu and Mullen, 2nd, *J. Biol. Chem.* 136, 339 (1940).

b—Hoffmann-LaRoche, Inc., Nutley, New Jersey.

c—Amino Acid Manufacturers, Univ. of California, Los Angeles, A.P. Grade.

d—Amino Acid Manufacturers, Univ. of California, Los Angeles, C.P. Grade.

e—Eastman Kodak, Rochester, New York.

partial rotations of the substituent groups has been attempted except for the mention of unpublished data on the dispersion of *d*-alanine in water by Levene and Rothen (7).

The partial rotations of the groups around an asymmetric center were derived from the rotatory dispersion curve, which was formed by plotting the reciprocal of the rotation against the wave length squared. If the resultant graph was a straight line, the dispersion was expressed by a one term Drude equation:

$$[\alpha] = A_0/(\lambda^2 - \lambda_0^2)$$

A_0 was a constant, λ the wave length under consideration, and λ_0^2 the wave length at which the curve crossed the zero axis. If the resultant graph was not a straight line, a two term Drude equation expressed the dispersion to a first approximation and usually within the precision of the measurements:

$$[\alpha] = A_1/(\lambda^2 - \lambda_1^2) \pm A_2/(\lambda^2 - \lambda_2^2)$$

The first term gave the partial rotation of the group having an anisotropic band at λ_1 and the second the sum of the partial rotations of the other groups. The second term could be considered as essentially the rotation of a single group, if this group had an anisotropic band (λ_2) much nearer the visible region of the spectrum than the anisotropic bands of the remaining groups. If the curvature of a dispersion given by a two term equation was not great, over a small portion of the spectrum it could be indicated by a one term equation. In this case the value obtained for λ_0^2 varied considerably depending on the segment of the spectrum being considered.

The value of λ_0 , obtained when a two term dispersion was represented by a single term in the visible region of the spectrum, could be correlated with values of λ_1 and λ_2 to indicate the sign of the partial rotations. The work of Hunter (12) which was summarized and applied by Levene and Rothen (13) indicated the following possibilities:

1. If $\lambda_2 < \lambda_0 < \lambda_1$:

Both terms had the same sign.

2. If $\lambda_2 < \lambda_1 < \lambda_0$:

Terms had opposite signs.

$$A_1/(\lambda^2 - \lambda_1^2) > A_2/(\lambda^2 - \lambda_2^2)$$

3. If

$$\lambda_0 < \lambda_2 < \lambda_1:$$

Terms had opposite signs.

TABLE II
Values of $\lambda_0(m\mu)$ Determined Graphically

	H ₂ O	1-HCl	1-NaOH	2-HCl	2-NaOH
<i>l</i> -Alanine.....	383	373	283	332	362
<i>d</i> -Valine.....	245	232	207	—	—
<i>l</i> -Leucine.....	Neg.	283	334	—	—
<i>l</i> -Phenylalanine.....	0	Neg.	An.	—	—
<i>l</i> -Tyrosine.....	—	Neg.	Neg.	—	—
<i>l</i> -Histidine.....	Neg.	An.	Neg.	—	—
<i>l</i> -Proline.....	126	170	200	—	—
<i>l</i> -Tryptophan.....	Neg.	An.	427	—	—
<i>l</i> -Aspartic acid.....	241	241	Neg.	—	Neg.
<i>l</i> -Glutamic acid.....	232	232	Neg.	—	340
<i>l</i> -Lysine.....	277	277	277	283	—
<i>l</i> -Arginine.....	265	270	249	249	—
<i>l</i> -Cystine.....	—	161	195	—	—
<i>l</i> -Methionine.....	Neg.	247	387	—	—

Note: Neg. indicates that λ_0^2 was negative; An. indicates anomalous.

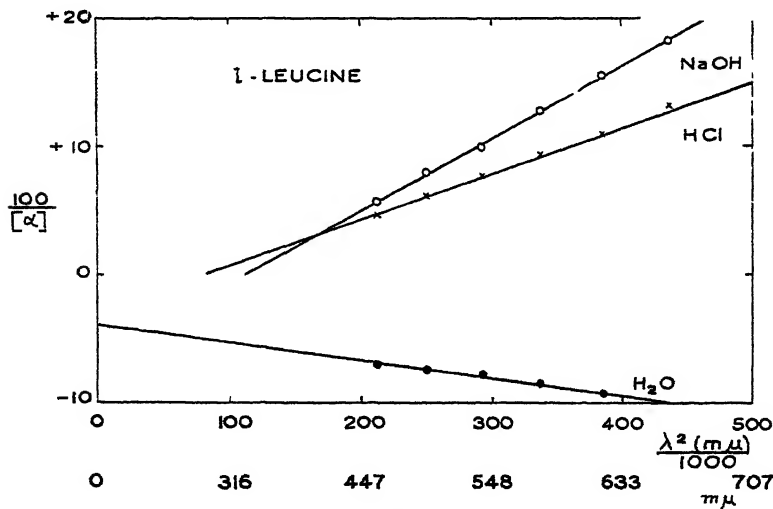


FIG. 1

The Rotatory Dispersion of *l*-Leucine and its Sodium and Hydrochloride Salt

$$A_1/(\lambda^2 - \lambda_1^2) < A_2/(\lambda^2 - \lambda_2^2)$$

In the last two cases the signs of the partial rotations were determined, for the term having the larger value from the analysis would have the same sign as was observed for the specific rotation in the visible.

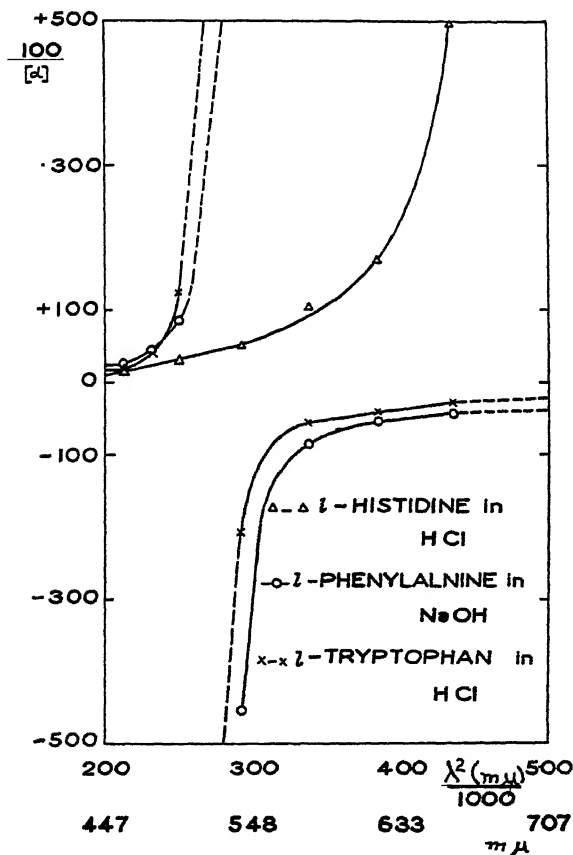


FIG. 2
Anomalous Dispersion of Certain α -Amino Acid Salts

The partial rotations of the various substituents about an asymmetric center were found to be of the same sign for all compounds belonging to the same homologous series and having the same configuration. Thus the relative configurations of the compounds followed directly from the determination of the partial rotations.

EXPERIMENTAL

The rotatory dispersion measurements were made on a Schmidt and Haensch (0.01°) polarimeter, which was calibrated from a standard quartz plate. The results have been summarized in Table I.

From the data in Table I dispersion curves of the amino acids were plotted with $100/[\alpha]$ against λ^2 . A representative dispersion curve is shown in Fig. 1. In all cases, except three, the dispersions formed a straight line and could be expressed by a simple Drude equation. The exceptions are shown in Fig. 2. On the basis of these curves, values of λ_0 were obtained graphically and tabulated in Table II.

DISCUSSION

An interpretation of the data in Table II depends on the position of the anisotropic bands of the carboxyl and amino groups, that is the value of λ_1 and λ_2 . The wave length of these bands can be approximated from the data available on the rotatory dispersion of carboxylic acids (13) and amines (14). The active bands of these functional groups overlap and cover the region of the spectrum between 140 and 205 $m\mu$. Therefore, without assigning λ_1 or λ_2 definitely to the carboxyl or amino group, it is possible to say that the value of each will be between 140 and 205 $m\mu$.

The values of λ_0 in Table II may now be compared with approximated values of λ_1 and λ_2 . This comparison divides the amino acids and their salts into distinct groups. Group one consists of those compounds having a value of λ_0 outside the region of the spectrum between 140 and 205 $m\mu$; group two consists of those compounds having a value of λ_0 in the region of the spectrum between 140 and 205 $m\mu$; and group three consists of compounds having an anomalous dispersion for which a value of λ_0 was not obtained.

Most of the amino acids and their salts that were studied are in the first group, and are examples of the second and third possibility indicated in the introduction. Thus, when $\lambda_2 < \lambda_1 < \lambda_0$ then the terms containing λ_1 and λ_2 are of opposite signs and the term containing λ_1 is numerically greater. The term containing λ_1 , therefore, has the same sign that was observed in the visible region of the spectrum. When $\lambda_0 < \lambda_2 < \lambda_1$, then the terms containing λ_1 and λ_2 are again of different sign. However, the term containing λ_2 is of greater magnitude and therefore has the same sign that was observed in the visible. The signs of the partial rotations of λ_1 and λ_2 found in this manner are tabulated in Table III. Thus, for *l*(+)-alanine the value of λ_0 was found to be

383 $m\mu$, which is greater than 205 $m\mu$ and therefore greater than λ_1 and λ_2 . Therefore, the dispersion of *l*(+)-alanine is of the second type, and the two terms expressing the dispersion are of different sign. The term containing λ_1 is numerically greater and has the same sign of rotation that was observed in the visible. Consequently, the sign of the term containing λ_1 is positive and that containing λ_2 the opposite, or negative.

The hydrochloride and sodium salts of cystine and proline are the only compounds in group two. These compounds also differ structurally from the others: for proline is the only amino acid studied that contains the α -amino group as part of a ring, and cystine is the only

TABLE III
Rotation in the Visible and Partial Rotations of Amino Acids

	H ₂ O			1-HCl			1-NaOH			2-HCl			2-NaOH		
	Visible	λ_1	λ_2	Visible	λ_1	λ_2	Visible	λ_1	λ_2	Visible	λ_1	λ_2	Visible	λ_1	λ_2
<i>l</i> -Alanine	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
<i>l</i> -Valine†	+	+	-	+	+	-	+	+	-						
<i>l</i> -Leucine	-	+	-	+	+	-	+	+	-						
<i>l</i> -Phenylalanine	-	+	-	-	+	-	An								
<i>l</i> -Tyrosine				-	+	-	-	+	-						
<i>l</i> -Histidine	-	+	-	An.			-	+	-						
<i>l</i> -Proline	-	+	-	-	(+)	(-)‡	-	(+)	(-)‡						
<i>l</i> -Tryptophan	-	+	-	An.			+	+	-						
<i>l</i> -Aspartic acid	+	+	-	+	+	-	-	+	-				-	+	-
<i>l</i> -Glutamic acid	+	+	-	+	+	-	-	+	-				+	+	-
<i>l</i> -Lysine	+	+	-	+	+	-	+	+	-	+	+	-			
<i>l</i> -Arginine	+	+	-	+	+	-	+	+	-	+	+	-			
<i>l</i> -Cystine				-	(+)	(-)‡	-	(+)	(-)‡						
<i>l</i> -Methionine	-	+	-	+	+	-	+	+	-						

† Results obtained on *d*-valine

‡ Borderline case

An indicates anomalous

α -amino acid studied that contains two asymmetric centers in the same molecule. The general values assigned to λ_1 and λ_2 , between 140 and 205 $m\mu$, are not of value in considering these cases. However, if the dispersions are analogous to those found for the compounds in group one, an arbitrary value of 205 $m\mu$ can be assigned to both λ_1 and λ_2 . When this is done the results are in accord with those of the first group and are tabulated in Table III with the notation that they are borderline cases.

In three cases the rotatory dispersion of the compounds studied, is anomalous. These compounds form the third group. The curves, Fig. 2, show a great similarity and in each case, as the observations are

made at decreasing wave length, the rotation proceeds from a negative value to ∞ and then from ∞ to a positive value. This follows the generalizations of Hunter (12) and should be true for all the α -amino acids of the *l*-series that have anomalous dispersions outside an absorption band.

The data from the analysis of the rotatory dispersion curves, in the manner of Hunter, and Levene and Rothen for the α -amino acids, leads directly to a configurational relationship. Thus all the compounds in group one show a positive sign of rotation for the term containing λ_1 and a negative sign for the term containing λ_2 . It can be generalized that all the α -amino acids and their sodium and hydrochloride salts having a carboxyl, amino, hydrogen, and methylene group attached to the asymmetric center will show a positive partial rotation for the anisotropic band nearest the visible, if the α -amino acid is of the *l*-series and gives a value of λ_0 which is not in the region of the spectrum between 140 and 205 $m\mu$. If the value of λ_0 falls between 140 and 205 $m\mu$, the generalization still holds, for the compounds studied, provided the arbitrary value 205 $m\mu$ is assigned to both λ_1 and λ_2 in the analysis for partial rotation. In the case of an anomalous dispersion an α -amino acid is assigned to the *l*-series if there is a change in sign of rotation from negative to positive as observations are made at decreasing wave length.

Up to this point no specific mention is made of the relationship between the partial rotation of the carboxyl and amino groups and that found for λ_1 and λ_2 . Although in the analysis it is not necessary that the partial rotation of these groups be connected to λ_1 or λ_2 it is interesting to note that Levene and Rothen (7) have suggested that the partial rotation of the carboxyl group is probably positive. This is based on the fact that most amino acids show a more positive rotation in acid solution than is found in alkaline solution.

The foregoing analysis indicates that the determination of the rotatory dispersion of α -amino acids affords a method using a minimum of sample for the determination of configuration. A few simple rules based on the nature of the curve produced when the reciprocal of the rotation is plotted against the wave length squared can be listed. The amino acid will belong to the *l*-series if:

1. the dispersion is normal, positive, and intercepts the zero axis in the region of the spectrum above 205 $m\mu$ squared;
2. the dispersion is normal, negative, and intercepts the zero axis in the region of the spectrum below 140 $m\mu$ squared;

3. the dispersion is anomalous and the sign of rotation changes from negative to positive with decreasing wave length.

The correlaries of these rules hold for compounds belonging to the *d*-series.

SUMMARY

1. The rotatory dispersions of fourteen α -amino acids as well as their sodium and hydrochloride salts have been found in the visible region of the spectrum.

2. An analysis of the rotatory dispersions has been made to indicate the usefulness of this measurement in the determination of configuration.

3. Simple rules have been indicated for the assignment of configuration to α -amino acids on the basis of rotatory dispersion curves.

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Some Reactions of Sodium Sulfide Added to Certain Tissues¹

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INTRODUCTION

The liver of a number of animals contains an enzyme system that, under certain conditions, converts cysteine to pyruvic acid, ammonia, and hydrogen sulfide (1). If the reaction is carried out in the presence of hydrogen sulfide containing radioactive sulfur, radioactivity is incorporated into the cysteine (2) showing that the sulfide sulfur is converted into cysteine sulfur. However, all attempts to start with pyruvic acid, ammonia, and hydrogen sulfide in the presence of the enzyme under anaerobic conditions and demonstrate a formation of cysteine by the Sullivan reaction (3) have failed. The failure may mean merely that the equilibrium for the reaction lies far to the side of the products. If this should be the case, one might expect that under aerobic conditions the energy necessary for the formation of cysteine could be furnished by some other reaction and, thus, an appreciable amount of cysteine formed. A difficulty with testing this possibility is that the sulfide added may inhibit the necessary oxidation. The fact that H_2S (and its salts) is toxic for intact animals is well known (4, 5), and the toxicity is, at least in part, due to inhibition of respiration. It is also well known that H_2S inhibits peroxidase (6), catalase (7) and cytochrome oxidase (8). Negelein (9) reported that the respiration of yeast cells was 70% inhibited by $10^{-5} M$ H_2S and completely inhibited by $10^{-4} M$. From the fact that iron-containing enzymes are inhibited, it is a common assumption that sulfide inhibits respiration by combining with one or more metal-containing enzymes in much the same way that cyanide is thought

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to do. As far as the writer is aware, there are no data available on the effects of adding sulfide to individual mammalian tissues. The work reported below was carried out to see what effect sulfide had on the *in vitro* respiration of liver, kidney, and brain tissues of the rat and conversely what effect the tissues had on the sulfide.

EXPERIMENTAL

Sodium sulfide added to the usual physiological solutions takes up oxygen. Whether the process represents a true autoxidation or a catalysis by traces of metals need not concern us here. Certainly, the oxidation is markedly accelerated by a number of metals (10). This fact makes interpretation of results obtained with tissues somewhat difficult, at least as far as rates of oxygen uptake are concerned, but it does not prevent some conclusions. Another limitation in working with sulfide is that alkali cannot be used to absorb CO_2 for it also absorbs H_2S .

Some experiments were carried out with cells of Fleischmann's bakers yeast suspended in primary phosphate solutions containing glucose. In agreement with Negelein it was found that small concentrations of H_2S inhibited respiration and permitted an aerobic fermentation rate equal to that under anaerobic conditions. This effect could be due in part to suppression of the Pasteur reaction (11), but the results indicated that respiration was also inhibited.

When slices of rat liver were substituted for the yeast cells, a very different result was obtained. Concentrations of added sulfide of the order of 10^{-5} *M* had no measurable effect. When greater concentrations were used, the respiration of the liver was not inhibited, but the oxygen uptake of the mixture was at least as great as the sum of the two separately.

The following experiment may be cited as a typical result. The two volume method of Warburg was employed. The same amount of liver, taken from a rat receiving food *ad libitum*, was used in each vessel. In one case it was suspended in 1.65 ml. of Ringer bicarbonate and in the other in 4.40 ml. No substrate was added. The gas space was filled with 5% CO_2 , 95% O_2 . The pH was 7.4. At the end of two hours, 567 cmm. of oxygen had been consumed and 622 cmm. of acid (CO_2 + fixed acid) produced. In other vessels the liver slice was omitted, but Na_2S was added to a concentration of 5×10^{-3} *M*. At the end of two hours, 327 cmm. of oxygen had been consumed and 173

cm. of acid produced. In other vessels both liver and Na_2S were added. At the end of two hours, 1012 cm. of oxygen had been consumed and 732 cm. of acid produced. At this point no H_2S could be recovered by aeration of the neutral or acidified solution from the vessels containing liver, and no iodine titratable material remained in solution.

The oxygen necessary to oxidize all of the added sulfide to sulfate was 1050 cm. The oxygen consumed by the mixture in excess of that consumed by the liver was 445 cm.

From the above figures alone there are a number of possible interpretations. The respiration of the liver could be inhibited until most of the sulfide was oxidized and then resume its usual course. The acid production does not indicate such an explanation, but might be compatible with it. The total oxygen consumed was almost sufficient to oxidize all of the added sulfide to sulfate. However, attempts to isolate the sulfate as barium sulfate in experiments like the one cited and in experiments on a larger scale showed quite clearly that such amounts of sulfate were not formed. We could not isolate sulfate equivalent to more than 10% of the sulfide added. Other evidence indicating that the liver respiration was not inhibited was obtained by adding a substrate that caused a change in the respiration of the liver (e.g. hexosediphosphate). A corresponding change resulted in the vessels containing liver plus H_2S .²

It is quite clear that the above results are very different from those obtained with yeast. One important difference in the experimental conditions was the pH of the solutions. The yeast cells were suspended in an acid medium so the Na_2S added was practically all converted to H_2S . The liver slices, however, were suspended in a medium buffered at pH 7.4, and since the first dissociation constant of H_2S is 9.1×10^{-8} , most of the sulfide added would be present as SH ion. The rate at which the SH ion can penetrate into a cell may be very different from that of H_2S so the interior of the liver cells may have been exposed to a smaller concentration of inhibitor than were the yeast cells, even though the total concentration of sulfide was much greater in the case of the liver. As one test of such an explanation, yeast cells buffered at pH 7.6 were

² An interesting example is the case of alanine. When liver plus H_2S used 294 cm. of oxygen, the addition of *dl*-alanine to a duplicate sample caused an increase to 427 cm. Presumably, the oxidation of alanine resulted in the formation of dehydroalanine. Should this compound rearrange to its isomer, α -amino acrylic acid, and add H_2S , cysteine would be formed. We were not able to demonstrate such a formation of cysteine by the Sullivan reaction under our conditions.

tested. They were still inhibited. However, since the permeability of yeast cells may be quite different from that of liver cells, it was thought that use of a cell-free liver extract might simplify the situation.

The livers were ground with twice their weight of saline or phosphate buffer and then centrifuged. The turbid supernatant layer was used directly. In a phosphate buffer without alkali present to absorb the CO_2 , such extracts showed a definite negative pressure on the manometer. This negative pressure was greatly increased when Na_2S was added. If we assume that the CO_2 production had not changed, the difference in negative pressure represents the difference in oxygen consumption. Whether or not this assumption about CO_2 is correct, the negative pressure represents oxygen consumption, and in many cases the values for oxygen consumed calculated from these negative pressures for extracts plus Na_2S were approximately equal to the sum of the values for extract and Na_2S separately. Thus, a freshly prepared extract showed a negative pressure corresponding to a consumption of 225 cmm. of oxygen in 140 minutes. A similar vessel containing 0.10 ml. of 0.096 M Na_2S but no extract consumed 216 cmm., and the mixture of the two consumed 429 cmm. After the extract had been heated at 90° for 15 minutes, it consumed only 11 cmm. of oxygen in 149 minutes, and the mixture of the heated extract plus Na_2S consumed 215 cmm. It is recognized that extracts are less sensitive to respiration inhibitors than are intact cells (12), but these extracts were readily inhibited by KCN. A concentration of $3 \times 10^{-3} M$ KCN in a phosphate buffer caused a 41% decrease in oxygen consumption while the same concentration of Na_2S caused a large increase with the same extracts. This finding need not imply any necessity for changes in our understanding of the mechanism of the oxygen uptake. The result can be explained in different ways with different assumptions.

Results obtained by adding different amounts of Na_2S to an extract and following the oxygen consumption are shown in Table I. It may be observed that the smallest concentration of Na_2S caused the greatest increase in oxygen uptake at the start, and that this increase continued until an amount of extra oxygen greater than that required to oxidize all the added sulfide to sulfate had been consumed. With the next greater concentration the extra oxygen was practically equivalent to that required for sulfate formation, but with the larger concentrations, it fell far short. In the case of the highest concentration, even the total oxygen consumption was not sufficient for complete formation of sulfate.

As in the case with liver slices only small amounts of sulfate could be isolated from experiments like the one in the last column, no sulfide could be recovered, and no iodine titratable material was present. It would seem reasonable to expect that some of the H_2S had been converted to free sulfur. Such an oxidation would require only one-fourth as much oxygen. However, the solutions did not show any visible sulfur precipi-

TABLE I

Effect of Na_2S on Oxygen Uptake of Liver Extract in Phosphate Buffer of pH 7.5

Time Minutes	Concentration of Na_2S Added in Mols per Liter				
	0	8×10^{-4}	2×10^{-3}	4×10^{-3}	8×10^{-3}
	mm. O_2 used*	mm. O_2 used	mm. O_2 used	mm. O_2 used	mm. O_2 used
15	51	74	71	64	58
30	115	157	151	133	121
45	167	237	229	202	181
60	190	276	288	261	239
90	233	332	380	394	336
120	263	366	426	491	410
180	291	405	477	559	585
240	314	438	512	600	687
300	336	466	542	634	731
mm. O_2 used in excess of liver alone		130	206	298	395
mm. O_2 required to oxidize sulfide to sulfate		86	215	430	860

* The figures given are calculated from the negative readings on the manometers. CO_2 production is not taken into account. The vessels had a volume of about 15 ml., and the volume of liquid used was 2.4 ml.

tate and when sulfur determinations were run on the material insoluble in HCl as described previously (1), very little (6 to 7% of the sulfur added as sulfide) was found. However, if at the end of the experiment on oxygen uptake, cadmium acetate was added to the inset and sodium thioglycolate to the untreated solution, rather large amounts of H_2S were formed. Thus, in seven experiments similar to that in the last column of Table I, an average of 30% of the added sulfide was recovered as H_2S after thioglycolate addition. In controls where cadmium acetate

was added to the inset but no thioglycolate was added, no trace of H_2S was obtained. Apparently, the solutions contained a soluble substance that reacted with thioglycolate to form H_2S . This result would probably be obtained if polysulfides were formed, but when the solutions were made strongly acid and allowed to stand, neither H_2S nor insoluble sulfur were formed. The substance responsible for this H_2S formation has not been further identified. It may be sulfur, present in some soluble or very stable colloidal form. At any rate, we shall consider it to be on the oxidation level of free sulfur and correct the total oxygen consumption by the amount required to produce the amount of sulfur found. The remaining oxygen, in excess of that consumed by the liver alone, could not oxidize more than an average of 45% of the sulfide added, to sulfate. Since 70% must be accounted for if all the sulfide disappeared by oxidation, then considerable amounts of partially oxidized sulfur must be present. Since the solutions did not reduce iodine, it seemed probable that any partially oxidized sulfur present would be in the form of polythionates. According to Kurtenacker and Goldbach (13), polythionates are broken down by alkali at 100° to thiosulfate and sulfite. Since both of these products titrate with iodine, the difference in iodine titration before and after heating with alkali is a measure of the polythionates present.

The method was applied to the trichloroacetic acid filtrates. One-half was treated with acetic acid and titrated directly. The other half was heated at 100° in 5% KOH for ten minutes, cooled, acidified with acetic acid, and titrated (14). Controls containing the tissue preparation but no added sulfide were run and the change obtained was applied as a correction to the experiments containing sulfide. In every case where sulfide was present, relatively large changes in iodine titration were obtained. In three experiments the production of H_2S with thioglycolate was run first and then the iodine titration before and after heating with KOH. In each case the results showed that the substances reacting in the two methods were independent of each other, *i.e.* the polythionates were not reduced to H_2S by thioglycolate, and the H_2S precursor did not reduce iodine after heating with alkali. If we assume that equal amounts of the S_3 , S_4 , and S_5 polythionates³ were present and were broken down in the manner stated by Kurtenacker and Goldbach, we

³ The need for separating the polythionates was not realized at the time the experiments were done. If it should turn out that only one or two of them were present, it would not change the conclusions in any way.

can calculate the amount of sulfur present as polythionates and the amount of oxygen necessary to produce it. An average of five experiments calculated in this way indicated that 54% of the added sulfide was present as polythionates. Correcting the oxygen consumption for the amount of oxygen required to form the polythionates, the remaining oxygen was sufficient to oxidize an average of 15% of the added sulfide to sulfate. The sum of the sulfur found as sulfur, as polythionates, and as sulfate (on basis of O_2 consumed) accounted approximately quan-

TABLE II
Recovery of Sulfur Added to Liver Preparations as Sodium Sulfide

	Liver extract	Liver slice
O_2 used by liver alone (cmm.)	322	217
O_2 used by liver + Na_2S (cmm.)	779	773
Sulfide added (mg. sulfur)	0.64	0.64
Sulfur formed (mg.)	0.20	0.14
O_2 Equivalent of sulfur formed (cmm.)	70	49
Polythionates formed: (difference in iodine titration due to heating with alkali) (ml. 0.01 N I_2)	1.45	1.40
Sulfur equivalent of polythionates (mg.)	0.37	0.36
O_2 Equivalent of polythionates (cmm.)	292	281
Remaining sulfide (mg. S)	0.07	0.14
O_2 required to oxidize remaining sulfide to sulfate (cmm.)	98	196
Extra O_2 used (<i>i.e.</i> O_2 available for sulfate formation) (cmm.)	95	226

titatively for the sulfide added. Examples of the data on which the calculations were based are given in Table II.

Some experiments were also carried out with kidney slices and kidney extracts. They indicated that kidney tissue behaves in much the same way as liver so the results are not given here.

Other experiments were done with brain slices and a few with brain extracts. The results obtained were quite different than those obtained with liver or kidney and more like those obtained with yeast. Results of a typical experiment obtained by the two volume method are shown in Table III. The gas exchange caused by the Na_2S alone, at the concentration and under the conditions used, was negligible and is not shown. It is apparent that the addition of Na_2S caused a large inhibi-

tion in the oxygen consumption of the brain slice and permitted a relatively large acid production. Smaller concentrations of Na_2S produced a correspondingly smaller change, but even $5 \times 10^{-5} M$ caused an appreciable inhibition of oxygen uptake and an increase in acid production. Anaerobic glycolysis was also inhibited by Na_2S even at $5 \times 10^{-4} M$, but it was far less sensitive than the respiration, and much larger concentrations of sulfide did not inhibit it entirely. With larger concentrations of sulfide under aerobic conditions not only did the gas exchange of the sulfide alone become appreciable, but a new and peculiar phenomenon appeared. Apparently the presence of brain tissue in-

TABLE III

Effect of Sodium Sulfide on the Metabolism of Brain Slices in Ringer-Bicarbonate-Glucose Solutions

Time	Brain alone		Brain + $5 \times 10^{-4} M \text{Na}_2\text{S}$	
	O_2 consumed	Acid produced (CO_2 + fixed acid)	O_2 consumed	Acid produced (CO_2 + fixed acid)
<i>min.</i>	<i>cmm.</i>	<i>cmm.</i>	<i>cmm.</i>	<i>cmm.</i>
30	83	93	22	67
60	142	145	53	116
90	221	220	93	168
120	243	239	108	188

The amount of tissue used was 138 mg. (initial wet weight). The gas space contained 5% CO_2 , 95% O_2 , the pH was 7.4 and the glucose concentration was 0.008 M .

hibited the oxidation of the sulfide, at least temporarily. This fact can be shown most clearly in a phosphate buffer, and a typical experiment is shown in Table IV. The figures are calculated from the negative pressures on the manometers without consideration of the CO_2 produced. In the case of brain the figures do not represent the true O_2 consumption but they do demonstrate the absence of oxygen consumption for the mixture. Readings for three different times are recorded for the brain and the Na_2S separately and for triplicate samples of brain plus Na_2S . It is apparent that even for the relatively high concentration of Na_2S employed the oxygen consumption was very low for a considerable period of time, but then became quite rapid. Such a result is typical. A

similar tendency may be observed in the oxygen consumption figures shown in Table III and may be contrasted with the results shown in Table I. The time at which the rapid uptake of oxygen began (or the time during which the oxygen uptake was inhibited) was by no means constant, and we were not able to control it at will. It may depend, to some extent at least, upon the metals present as impurities. In the experiments cited no special precautions were taken to remove metals, but the experiments were prepared with reasonable care. The chemicals used were commercial reagent grade, and the vessels employed were cleaned in a bath of hot sulfuric-nitric acid mixture.

TABLE IV

Apparent Oxygen Consumption of Brain, of Sodium Sulfide, and of Brain Plus Sodium Sulfide

Time <i>min.</i>	Brain alone	$1.3 \times 10^{-2} M$ sodium sulfide	Brain + $1.3 \times 10^{-2} M$ sodium sulfide (in triplicate)		
	<i>mm. O₂</i> <i>used*</i>	<i>mm O₂ used</i>	<i>mm. O₂</i> <i>used</i>	<i>mm. O₂</i> <i>used</i>	<i>mm. O₂</i> <i>used</i>
85	26	37	0	0	1
130	37	61	31	6	5
200	53	104	162	93	113

The experiments were carried out in a Ringer-phosphate-glucose solution of pH 7.4 with air in the gas space.

* The figures are calculated from the negative readings on the manometers without consideration of the CO₂ produced.

It might be supposed that the onset of rapid oxygen uptake corresponded to the removal of most of the Na₂S by some non-oxidative mechanism, but the results indicated that such was not the case. In an experiment like the one recorded in Table IV, after the rapid oxygen uptake was well started, 20% of the added sulfide was recovered as H₂S by aeration. The amount recovered was ample to cause inhibition when it was added at the start. A possible explanation of such results is that the brain tissue combined with the traces of metals necessary as catalysts for the oxidation of sulfide and later, as a result of changes undergone during the experiment, gradually releases them.

As in the liver experiments, some polythionates and some sulfur were formed in the brain plus sulfide experiments. Under our conditions

polythionates were formed from Na_2S in the absence of any tissue. The presence of liver caused at least a three-fold increase in the amount formed, while brain caused a somewhat smaller increase. It should not be concluded, however, that either liver or brain contains an enzyme specifically concerned with the formation of polythionates.

SUMMARY

Sodium sulfide was added to slices and extracts of rat liver, kidney, and brain and the effect on oxygen consumption and glycolysis observed.

The respiration of liver preparations was not inhibited by Na_2S addition. The oxygen consumed by the mixture was at least as great as the sum of that consumed by the two separately. Relatively large amounts of added sulfide were completely removed by conversion to sulfur, polythionates, and sulfate, without apparent effect on the respiration of the liver preparations.

Kidney tissue behaved toward sodium sulfide much the same as did liver.

The oxygen consumption of brain slices was readily inhibited by the addition of sodium sulfide, and the aerobic glycolysis was increased. A concentration of $5 \times 10^{-4} M$ sulfide caused a 75% decrease in the oxygen consumed by brain during the first thirty minutes. The anaerobic glycolysis of brain was also inhibited by sulfide, but much less readily than the respiration. Brain tissue also inhibited the oxidation of sodium sulfide, at least temporarily, so a mixture of the two consumed much less oxygen than either one alone.

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CO₂-Fixation and Succinic Acid Formation by a Cell-free Enzyme Preparation of *Escherichia coli*¹

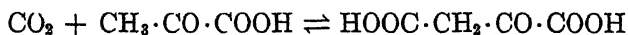
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INTRODUCTION

Since 1935, when the concept of CO₂-utilization in heterotrophic metabolism was first established (Wood and Werkman, 1935), and 1938, when the relationship was demonstrated between CO₂-utilization and succinic acid formation (Wood and Werkman, 1938), considerable progress has been made towards an understanding of the fundamental rôle of CO₂-fixation in heterotrophic metabolism. The fixation reaction has been demonstrated with a number of heterotrophic bacteria, with yeasts, molds, and trypanosomes, and with plant and animal tissue (cf. Werkman and Wood, 1942). The use of the isotopes, radioactive (C¹⁴) and the stable (C¹³) has confirmed the occurrence and extended our knowledge of this reaction. Attempts to further elucidate the mechanism of the fixation through the use of enzyme systems have only recently been inaugurated. Krampitz and Werkman (1941, 1942), Werkman, *et al.* (1942), and Krampitz, *et al.* (1943) using an acetone preparation of *Micrococcus lysodeikticus*, presented the first direct evidence that oxalacetic acid is a component of the fixation reaction, and that the reaction



is reversible. Evans, *et al.* (1942), working with a cell-free liver preparation, obtained fixation of CO₂, but did not trace the radioactive carbon. A preliminary communication (Kalnitsky and Werkman, 1942) reported the preparation of a cell-free enzyme system from *Escherichia coli* which fixed C¹³O₂ in the carboxyl group of succinic acid. Quantitative data for the fixation reaction with this enzyme system are presented. An actual net uptake of carbon dioxide was observed with pyruvate as

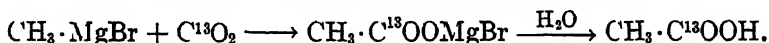
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substrate, and the fixed $C^{13}O_2$ was located in the carboxyl groups of succinic, formic, and lactic acids. Another mechanism for succinic acid formation besides that of CO_2 -utilization is also demonstrated with this enzyme preparation, in which the condensation of $CH_3 \cdot C^{13}OOH$ is established. The mechanisms for the formation of succinic and formic acids are discussed.

METHODS

Preparation of the enzyme system and the analysis and isolation of the fermentation products have already been described (Kalnitsky and Werkman, 1943). Heavy carbon (C^{13}), was used as a tracer in the present experiments, and in order to determine the C^{13} content of the compounds isolated, they were converted to CO_2 by oxidation with persulfate (Osburn and Werkman, 1932). The CO_2 evolved was collected in 1.5 *N* carbonate-free NaOH, then liberated by acidification, and the C^{13} content determined by mass spectrometer analysis (Nier, 1940).

The $CH_3 \cdot C^{13}OOH$ was prepared by the Grignard reaction:



EXPERIMENTAL

Pyruvate is attacked anaerobically by the enzyme preparation, and carbon dioxide is evolved in manometric experiments with bicarbonate present (Kalnitsky and Werkman, 1943). Occasionally, however, the carbon dioxide evolution fell far short of the usual values, although identical amounts of pyruvate had been fermented. Such results seemed to point to the utilization of carbon dioxide, perhaps under conditions not optimal, therefore experiments were carried out with different gaseous atmospheres (Table I). The CO_2 evolved during the course of the fermentation and the residual CO_2 evolved on acidification after completion of the fermentation equals the total CO_2 . Column 4 gives the CO_2 obtained by simultaneously tipping the pyruvate and sulfuric acid into the Warburg vessel containing the enzyme preparation and the bicarbonate. The difference between Columns 3 and 4 represents the carbon dioxide produced, or utilized in the fermentation. It is seen, that under a nitrogen atmosphere, 107 μ l. of carbon dioxide were produced during the course of the fermentation. Under an atmosphere of hydrogen, much less carbon dioxide is produced, and in one instance,

there was a slight loss. However, under atmospheres of 5% and 10% carbon dioxide in hydrogen, carbon dioxide was consistently unaccounted for in every case. The amount of CO₂ utilized was smaller under an atmosphere of 10% CO₂ in N₂, whereas increasing the percentage of

TABLE I
Effect of Different Gases on Fixation of CO₂ by E. coli Juice

Atmosphere	1	2	3	4	CO ₂
	CO ₂ evolved (μl)				
	During fermentation	On acidifying after fermentation	Total (1 + 2)	On acidifying before fermentation	
N ₂	332	819	1151	1044	+107
H ₂	335	841	1176	1171	-5
	363	862	1225	1245	+20
5% CO ₂ in H ₂	308	950	1258	1412	-154
	318	940	1258	1412	-154
10% CO ₂ in H ₂	420	1158	1578	1670	-92
	426	1118	1544	1670	-126
	381	1187	1568	1720	-152
	340	1202	1542	1720	-178
	370	1138	1508	1720	-212
10% CO ₂ in N ₂	477	1105	1582	1634	-52
50% CO ₂ in H ₂	459	1624	2083	1969	+114
	463	1610	2073	1969	+104
100% CO ₂	667	2296	2963	2888	+75
	610	2290	2909	2888	+21

Each cup contained 0.8 ml. juice, 0.022 *M* pyruvate, and 0.04 *M* NaHCO₃. Total volume, 2.3 ml.; time, 3 hrs.; temp., 30.4°C.

carbon dioxide to 50% and 100%, resulted in a net production of CO₂. Variation of the concentration of either the juice, the bicarbonate, or the pyruvate invariably brought about a much smaller net utilization of CO₂, and frequently caused a net production of CO₂.

The contents of the five experimental cups under an atmosphere of 10% carbon dioxide in hydrogen were carefully rinsed out and the

precipitated proteins filtered off. Volatile acids were removed by steam distillation, and the residue was extracted with ether overnight. The ether extract was tested for the presence of succinic acid with a succinic dehydrogenase preparation obtained from beef heart. Succinic acid found amounted to 760 μ l. (on the basis that 2 moles of succinate take up 1 mole of O_2 , and that 1 mM. succinate is equivalent to 22,400 μ l.).

TABLE II

*Per Cent C^{13} in Products of Pyruvate and $NaHC^{13}O_3$ Dissimilation by *E. coli* Enzyme Preparation*

Fermentation No	1		2	
	mM	% excess C^{13}	mM	% excess C^{13}
Original $NaHCO_3$	3.966	5.10	5.295	5.10
Residual $NaHCO_3 + CO_2$	4.178	4.68	5.465	4.17
Pyruvate fermented	2.85		2.67	
Products				
CO_2	0.212		0.17	
Formic	2.30	0.16	1.85	0.08
Acetic	2.30	0.00	2.03	0.00
Lactic (COOH)	0.07	0.61	0.10	0.27
Succinic	0.24		0.383	
Succinic (COOH) $_2$		0.42		0.65
Succinic (CH $_2$) $_2$		0.00		0.00

Fermentation No. 1: Contained 30 ml. enzyme preparation; $NaHC^{13}O_3$, 0.05 M; pyruvate, 0.05 M; phosphate, 7 ml. (0.1 M, pH 6.2); total volume, 75 ml; atmosphere, $C^{13}O_2$ in H_2 ; time, 4 hours; temp., 30.4°C.

Carbon recovery 96%; O/R index, 1.03.

Excess C^{13} recovered, 100%.

Fermentation No. 2: Contained 38 ml. enzyme preparation; $NaHC^{13}O_3$, 0.07 M; pyruvate, 0.04 M; phosphate, 10 ml. (0.1 M, pH 6.2); total volume, 75 ml; atmosphere, $C^{13}O_2$ in H_2 ; time, 4 hours; temp., 30.4°C.

Carbon recovery, 99%; O/R index, 0.97.

Excess C^{13} recovered, 87%.

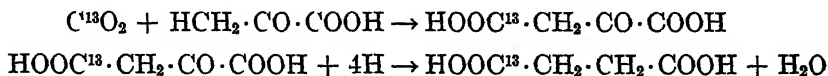
The CO_2 unaccounted for also was 760 μ l. Thus carbon dioxide was fixed and succinic acid formed in equimolar amounts.

Accordingly, with the indication that carbon dioxide is fixed and succinic acid is formed in equivalent amounts, experiments were carried out on a larger scale with C^{13} in the bicarbonate of the medium. Pyruvate and $NaHC^{13}O_3$ were placed in a 300 ml. Erlenmeyer flask which was connected to a condenser, and then to a bead tower containing 15 ml.

of 1.5 N CO₂-free NaOH. The whole system was put under a slight vacuum and the air above replaced with CO₂-free H₂. Acid phosphate was added to liberate some C¹³O₂ and to give an atmosphere of C¹³O₂ in H₂. The enzyme was then added. After completion of the fermentation, the proteins were precipitated and residual CO₂ liberated by the addition of 10 ml. of 20 per cent metaphosphoric acid. The solution was refluxed and aerated to complete the removal of CO₂. The resulting CO₂-free mixture was filtered, and from the filtrate the fermentation products were isolated and analyzed for the presence of excess C¹³. Results are shown in Table II. The concentration is expressed as per cent C¹³ in excess of the normal complement of C¹³, *i.e.*, the per cent in excess of 1.09. The bicarbonate is the only source of carbon containing C¹³ in excess of 1.09, and the variation in the mass spectrometer analysis of C¹³ is ± 0.02 . The excess C¹³ values of the succinic, formic, and lactic acids are significant and indicate fixation of CO₂.

Mechanism of Succinic Acid Formation

The succinic acid isolated from the fermentation was converted to a mixture of fumarate and malate, by means of a succinic dehydrogenase preparation obtained from beef heart. The malate was then oxidized with KMnO₄ to acetaldehyde plus 2 CO₂. The two carbon dioxide molecules originate from the two carboxyl groups, and acetaldehyde from the methylene groups of the original succinic acid (Wood, *et. al.*, 1941). The acetaldehyde was then oxidized to CO₂ with potassium persulfate (Osburn and Werkman, 1932), and the C¹³ content of the carboxyl and of the methylene carbons determined. The C¹³ content of the methylene carbons of succinic acid was found to be normal (1.09%), whereas the values for the carboxyl groups showed that a significant amount of excess C¹³ was present. The results conclusively indicate that there has been fixation of carbon dioxide by the enzyme preparation of *Escherichia coli* with pyruvate as substrate, and that the fixed carbon is located in the carboxyl groups of the succinic acid formed. The values for the carboxyl carbons are not high enough to indicate that all of the succinate has been formed by a fixation of CO₂. If the fixed C¹³O₂ is present in only one of the carboxyl groups of the succinic acid, according to the reactions:



(Wood, *et al.*, 1941), the excess C^{13} in the single carboxyl group containing fixed C^{13} will be 0.84 and 1.30, *i.e.*, twice as large as the value for the two carboxyl groups (Table II). These values are much lower than the excess C^{13} in the bicarbonate (4.68 and 4.17), and it is evident the carboxyl was not derived solely from this source. Assuming that the $C^{18}O_2$ is in equilibrium with the $C^{13}O_2$ in the medium and at the enzyme surface, then, if all the succinate were formed by a fixation of CO_2 , the C^{13} content in the carboxyl group of the succinate should be at least as large as the C^{13} content in the bicarbonate at the end of the fermentation. Since this was not so, either the isotopes are not at equilibrium at the enzyme surface, or there is another mechanism of succinic acid formation. It will be recalled that in the manometric experiments (Table I) the succinate was formed in amounts equimolar to the CO_2 utilized, whereas in the larger experiments (Table II), there was a small net production of CO_2 during the course of the fermentation. In order to

TABLE III

C^{13} in Succinate Formed in Quantities Equimolar to Carbon Dioxide Utilized

CO_2 Missing $\mu l.$	Succinate formed $\mu l.$	% Excess C^{13} in total succinate	% Excess C^{13} in carboxyl groups, calculated
1311	1368	0.21	0.42

Each cup contained juice, 0.8 ml.; pyruvate, 0.024 M; $NaHC^{13}O_3$, 0.046 M; phosphate, 0.2 ml (0.1 M, pH 6.2); H_2SO_4 (1:1), 0.3 ml.; (side arm); total volume, 2.3 ml.; atmosphere, $C^{14}O_2$ in H_2 ; time, 3 hours.

ascertain whether succinate contains a high complement of fixed C^{13} when it is formed in quantities equimolar to the CO_2 utilized, small manometric experiments were set up with $NaHC^{13}O_3$, and the C^{13} of the isolated succinic acid was determined (Table III). The quantity of 1311 $\mu l.$ CO_2 was utilized in eleven Warburg cups, and 1368 $\mu l.$ succinate were formed. However, the excess C^{13} content of the carboxyls of the succinate formed (0.83) was not higher than that obtained in large-scale experiments (0.84 and 1.30), where no net loss of CO_2 occurred. Here again, though the amount of succinate formed, corresponds to the CO_2 utilized, the C^{13} content of the resulting succinate indicated that there was another mechanism for succinic acid formation besides that of CO_2 utilization.

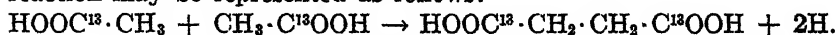
Acetic Acid Condensation

Thunberg (1920) and Wieland (1922) suggested the formation of succinic acid by a dehydrogenation of two moles of acetic acid. Since

then, numerous investigators have presented evidence for this mechanism of succinic acid formation by yeasts (Wieland and Sonderhoff, 1932), molds (Butkevich and Fedorov, 1929, 1930), bacteria (Wood and Werkman, 1936; Slade and Werkman, 1943), and animal tissue (Weil-Malherbe, 1937). Sonderhoff and Thomas (1937), investigated the metabolism of yeast using trideuteroacetic acid, and found deuterium in the succinate formed. The amount of deuterium in the succinate was less than that in the acetate. Nevertheless, their experiments indicated that succinate was derived from acetate, although the mechanism is probably more complex than simple dehydrogenation. Kleinzeller (1941), reported that yeast did not form succinate from acetate under the conditions of his experiments, and Foster, *et. al.* (1941), state that "the evidence for a synthesis of succinate through two acetates (Thunberg-Wieland condensation) has never been conclusive."

Confirmation of Sonderhoff and Thomas' work was necessary and was presented by Slade and Werkman (1943). These authors, working with *Aerobacter*, and using acetic acid containing C^{13} , obtained definite evidence for the formation of succinic acid by the condensation of two C_2 molecules, probably those of acetic acid.

On the basis that acetic acid condensation might be a mechanism of succinate formation with the enzyme preparation of *Escherichia coli*, a large-scale experiment similar to those described in Table II was carried out with pyruvate and $CH_3 \cdot C^{13}OOH$ as substrates. Table IV gives the C^{13} content of the products of this dissimilation by the *E. coli* enzyme preparation. The only source of excess C^{13} was the added acetate. Succinic acid was the only compound other than acetic acid which was found to contain excess C^{13} . The heavy carbon of the succinic acid was located exclusively in the carboxyl groups. Since the residual CO_2 of the fermentation contained no excess C^{13} , it is evident that the heavy-carbon succinate was not formed by the fixation reaction. Therefore, this experiment conclusively shows that, besides the fixation reaction for succinate formation, the preparation has a second mechanism, the condensation of acetic acid (or its derivative) with a two or three-carbon molecule (pyruvic acid or its derivative). The over-all reaction may be represented as follows:



The present evidence does not establish that it is specifically two molecules of acetic acid that react, but it does establish that acetic acid can be converted to succinic acid by condensation of two molecules of acetic acid or by a reaction involving acetic acid. Slade and Werkman

(1943) have also demonstrated with *Aerobacter* the reverse reaction, *i.e.*, the cleavage of succinate to acetate. This evidence supports the proposal of the formation of succinate by a C_2 plus C_2 addition, rather than by a 2-carbon plus 3-carbon addition. The enzyme system reported here apparently does not possess this cleavage mechanism, since, in the presence of $NaHC^{13}O_3$ (Table II), the succinic acid contained an excess of C^{13} , whereas the C^{13} content of the acetic acid was normal.

Since two mechanisms for succinic acid formation have been demonstrated with this preparation, the question arises as to the percentage

TABLE IV

*Per Cent C^{13} in Products of $CH_3 \cdot C^{13}OOH$ and Pyruvate Dissimilation by *E. coli* Enzyme Preparation*

	mM.	% Excess C^{13}
$CH_3 \cdot C^{13}OOH$ added.....	2.06	1.30
Pyruvate fermented.....	2.98	
<i>Products</i>		
CO_2	0.30	0.01
Formic.....	2.14	0.02
Acetic.....	4.27	0.65
Lactic.....	0.07	0.02
Lactic (COOH).....		0.02
Succinic.....	0.392	0.13
Succinic (COOH) $_2$		0.26
Succinic (CH $_2$) $_2$		0.02

Fermentation contained pyruvate, 0.038 M; $CH_3 \cdot C^{13}OOH$, 0.024 M; $NaHCO_3$, 0.064 M; phosphate, 2 ml. (0.5 M, pH 6.2); enzyme preparation, 30 ml.; total volume, 85 ml.; atmosphere, CO_2 in H_2 ; time, 4 hours; temp., 30°C.

Carbon recovered, 97%; O/R index, 1.05.

Excess C^{13} recovered, 107%.

of the succinate formed by each mechanism. The quantitative isotopic data can be used to give some information, although there are limitations that have to be considered. The calculations are not exact because of uncertainty as to mechanisms of the reactions, assumption of equilibria between the C^{13} and C^{12} compounds at the enzyme surface, and the difficulty in determining the concentration of the C^{13} compounds during the course of the fermentation. Minimal values can be calculated but the calculation of maximal or exact values involves assumptions.

The acetate added at the beginning of the experiment (Table IV) contained 1.30 per cent excess C^{13} . If the C^{13} content of these acetic

acid molecules is not diluted by acetic acid arising from pyruvate, and, if condensation takes place between two acetic acid molecules, each molecule of succinate formed will contain 1.30 per cent excess C^{13} , *i.e.*, the same percentage as the added $CH_3 \cdot C^{13}OOH$. The experimental value for the succinate is 0.13% excess C^{13} . If X = the per cent of succinate formed by condensation, then, if X moles of succinate containing 1.30% excess C^{13} are diluted with ordinary succinate to 100 moles, the resulting succinate will contain 0.13% excess C^{13} ($1.30 \times X = 100 \times 0.13$). The value for the succinate formed by condensation of $CH_3 \cdot C^{13}OOH$ containing 1.30% excess C^{13} is therefore 10%. This is the lowest possible value for acetate condensation which could give the experimental value of 0.13%.

If the succinate arose by condensation of one molecule of added acetate with one molecule of pyruvate, the excess C^{13} content of the resulting succinate will be one-half the acetate, or 0.65%. The succinate formed by condensation will be 20% in this case ($0.65 \times X = 100 \times 0.13$).

The above calculations are not exact for it is apparent that acetate arising from the pyruvate will also condense. Thus, the acetate which enters the condensation reaction will have a C^{13} concentration less than that of the original added acetate, but somewhat greater than that of the final acetate. The per cent succinate formed by condensation as calculated on the basis of the final acetate is probably a maximal value. If both portions of the succinate come from the final acetate, the value is 20% ($0.65 \times X = 100 \times 0.13$). If the succinate is formed by acetate and pyruvate condensation, the calculation on the basis of the C^{13} content of the final acetate gives 40% ($0.325 \times X = 100 \times 0.13$).

On the same basis, and assuming the CO_2 is fixed in only one carboxyl group (*cf.* Krampitz, *et al.*, 1943), the per cent of succinate arising from CO_2 fixation can be calculated to be between 16.5 ($5.10 \times X = 100 \times 0.84$) and 18% (Fermentation No. 1, Table II) and between 25 and 31% (Fermentation No. 2, Table II).

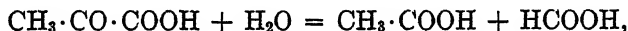
Even if the maximal values are taken for the per cent of succinic acid formed by CO_2 utilization and acetic condensation, only 71% of the succinate mechanism is accounted for. The results were not from the same fermentation and there may have been some variation. We have no assurance, however, that the assumptions on which these calculations are based are entirely correct. For example, the $C^{12}O_2$ produced at the enzyme surface may be more closely bound to the enzyme and

therefore may be preferentially fixed, even though both may be in equilibrium in the medium. There is no direct evidence that the added $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$ comes to equilibrium with the acetate formed from pyruvate, or with an intermediate two-carbon molecule which is actually condensed to form succinic acid. Finally there is the possibility that there may be at least one more mechanism of succinic acid formation by this enzyme preparation, besides the two indicated above.

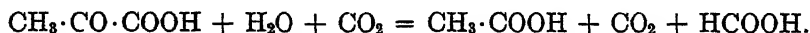
The principal point to be made is that there are at least two mechanisms of succinate formation which are of quantitative significance. Therefore, further investigation is necessary in cases where only one mechanism for succinate formation is postulated (Krebs and Eggleston, 1941).

Mechanism of Formic Acid Formation

At present there are two concepts for the formation of formic acid from pyruvate: (1) The hydroclastic split, in which pyruvic acid breaks down to acetic and formic acids,



and (2) Reduction of CO_2 by either gaseous hydrogen or an organic hydrogen donator. Neuberg (1914) showed the conversion of pyruvate to acetic and formic acids. He pictured the over-all reaction as a hydroclastic split of pyruvate to acetic and formic acids but also proposed a mechanism for the formation of formic acid by the reduction of carbonic acid (from pyruvate) by nascent hydrogen arising from the water in the medium. Tikka (1935) proposed the transformation of pyruvic acid by *E. coli* entirely without decarboxylation, through a hydroclastic split into equimolar amounts of acetic and formic acids. Krebs (1937) proposed that formate was not necessarily formed from the carboxyl group of pyruvic acid, but could arise from the CO_2 and water of the medium, according to the equation



His point was that this mechanism was possible, and the hydroclastic split was not definitely proved. Werkman and Wood (1942) stated that it was generally accepted that formic acid originates from pyruvic acid by a hydroclastic split. However, these authors pointed out the possibility that CO_2 could be reduced by an organic hydrogen donator to form formic acid.

Woods (1936) has shown that the reduction of CO_2 to HCOOH with H_2 occurs with *E. coli* and presumably with other bacteria that contain hydrogenlyase. The *E. coli* enzyme preparation used in these experiments was obtained from cells which were grown under strong aeration, with no formate present, and consequently, did not contain any hydrogenlyase when tested (Yudkin, 1932). In the presence of C^{18}O_2 , the absence of an excess of heavy carbon in the formic acid formed from pyruvate dissimilation would indicate that formic acid was not formed by reduction of CO_2 as such. The results of such experiments are presented in Table II. It is evident that the per cent of heavy carbon present in the formic acid in both fermentations is significant, but only slightly above the normal. Bearing in mind the fact that, with hydrogenlyase present, the C^{13} content of the formic acid formed is equal to the C^{13} content of the residual bicarbonate (Slade, *et. al.*, 1942), it is evident that when no hydrogenlyase is present, as in this instance, very little of the formic acid is formed by a reduction of CO_2 from the medium. Calculating the per cent of formic acid arising from a reduction of CO_2 , according to the C^{13} content of the formic acid formed and of the residual and original bicarbonate (Table II), we find that in Fermentation No. 1, from 3.2 to 3.5% of formate originated in this way, and in Fermentation No. 2, 1.2 to 1.8% of the formate was formed by a reduction of CO_2 from the medium. A slight residual hydrogenlyase activity might account for the small excess amount of C^{13} present in the formic acid. On the other hand, the C^{12} formed from pyruvate may be more closely bound to the enzyme surface and thus may be preferentially reduced. In this case, however, the formation of formic acid would not be the result of the reduction of CO_2 from the medium.

It appears that the reduction of CO_2 is probably not the mechanism by which formic acid arises in this case. However, the hydroclastic split, as pictured, is probably incorrect also, since phosphate is known to be necessary for this reaction (Kalnitsky and Werkman, 1943), and for the conversion of pyruvate into acetate, CO_2 , and molecular hydrogen (Koepsell and Johnson, 1942).

Lactic Acid Formation

In the dissimilation of pyruvic acid, very small amounts of lactic acid were formed. However, a significant amount of heavy carbon was fixed in the lactic carboxyl group in the presence of $\text{NaHC}^{18}\text{O}_3$. These experiments confirm previous work done with whole cells, where, concu-

rent with CO_2 utilization in succinic acid, heavy carbon was also found in the carboxyl group of the lactic acid produced by many organisms (Wood, *et al.*, 1942; and Slade, *et al.*, 1942). These results suggest that a C_4 dicarboxylic acid, formed by a C_3 and C_1 addition, may be a precursor to the formation of lactic acid. However, from the results in Table IV, it can be seen that succinic acid itself is probably not an intermediate in the formation of lactic acid. The succinic acid molecule contained an excess of heavy carbon (derived from the $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$), whereas the C^{13} content of the lactic carboxyl group was practically normal.

In view of the small amounts of lactic acid formed by this system, it would be inadvisable to try to formulate a mechanism for its formation, on the basis of the data in this paper. The mechanism of this reaction would probably be studied to greater advantage, by using an organism or an enzyme system producing much larger amounts of lactic acid.

SUMMARY

The dissimilation of pyruvate has been investigated using an active, cell-free enzyme preparation obtained from *E. coli*. The products are succinate, lactate, acetate, formate, and CO_2 . CO_2 was utilized and by the use of tracer experiments with the C^{13} isotope, fixed C^{13}O_2 was located in the carboxyl group of the succinic acid formed.

CO_2 fixation is not the only mechanism of succinic acid formation by this enzyme system. On addition of $\text{CH}_3\cdot\text{C}^{13}\text{OOH}$, succinic acid was isolated, containing excess C^{13} exclusively in the carboxyl groups. Therefore, condensation of acetic acid, or its derivative with a 2-carbon or 3-carbon molecule is another mechanism for the formation of succinic acid.

It is shown that in the absence of hydrogenlyase, the reduction of CO_2 from the medium is not the mechanism of formic acid formation.

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BOOK REVIEWS

Textbook of Biochemistry. By ROGER J. WILLIAMS, Professor of Chemistry in the University of Texas. D. Van Nostrand Co., Inc., New York, N. Y., 1942. x + 533 pp. \$4.00.

This is the second edition of the successful text which appeared in 1938. The same plan of presentation has been carried over into this edition; the distinctive contribution of the revision lies in the incorporation of considerable new information, particularly in the fields of intermediate metabolism and of the chemistry of the vitamins and the enzymes.

The text is designed for students of animal biochemistry and as such is suitable for medical students and for students in biochemistry interested in nutrition and animal physiology and chemistry. It could also be read with profit by the layman interested in the "why" and "how" of nutrition in these days of food rationing. Part I deals with the biochemical materials—proteins, lipides, carbohydrates and inorganic constituents—and includes a chapter on the colloidal properties of matter. Part II describes the composition of animal tissues and includes many tables of well selected data. The mineral, protein, carbohydrate, lipide and vitamin requirements of man and the experimental animals are presented in Part III. The last two parts deal with the bodily mechanisms for promoting and regulating chemical changes and with the metabolism (both anabolism and catabolism) of foods. An interesting final chapter on chemotherapy treats of the problems and latest advances in such lines as the arsenicals, drugs for sleeping sickness, quinine and other antimalarials, the sulfa drugs.

The material is presented in a lucid and interesting style. The findings of specialized researches are presented as final results only but sufficient citations are indicated to permit an interested student to read further. From a teaching viewpoint this procedure has the advantage of a development of the story without the distraction and the confusion of many citations and the flux of thought incidental to the final conclusion. The author appears to use good judgment in the interpretation of current trends in biochemistry and nutrition. In a few places the reviewer found statements which he might question but the differences could be settled only by a longer discussion than is warranted in a text of this sort.

The emphasis throughout is on the animal side of biochemistry, materials found in the plant world are noted only in so far as they enter into animal metabolism. The findings of mammalian physiology are used to explain the body's control of temperature, respiration and permeability and in the problem of absorption.

A glossary of specialized terms and the selected references at the end of most chapters will extend the usefulness of this very readable text.

W. M. SANDSTROM, St. Paul, Minn.

Biochemistry and Morphogenesis. By JOSEPH NEEDHAM, F.R.S. Sir William Dunn Reader in Biochemistry and Fellow of Gonville and Caius College, Cambridge: At the University Press. New York: The Macmillan Company, 1942. XVI + 785 pp. Price \$12.50.

It is impossible to rate too highly the merits of Needham's new volume on the biochemistry of developmental processes. The book has to be considered not merely as an up-to-date supplement of his fundamental *Chemical Embryology* published 10 years ago. Raised into a higher level of integration, it represents a pioneer journey into vast new domains of the fascinating border-land between biochemistry and morphogenesis "bringing what help it can" to both disciplines. No longer restricting himself to a critical survey of all existing knowledge concerning the raw materials and the metabolism of the developing organism, the author has adopted a more dynamic view, endowing the dualistic concept of morphogenetic stimulus versus tissue reaction with the role of organizing the tremendous amount of facts into a hierarchical order of increasing complexity. The work thus offers the unique spectacle of the "induction" of a biochemical mind by modern embryological ideas, and there is no doubt, the versatile competence of the author has rendered this experiment a full success.

In view of the vast scope of the book which incorporates in its bibliography some 7000 papers it is impossible to appraise it here from its manifold angles. After having given a detailed account of the constitution of the various egg types, of the manners of embryonic nutrition and protection, and of the effects of environmental factors upon embryogenesis, the author, in the main part of his volume, discusses in a hitherto unsurpassed completeness the three most important types of morphogenetic stimuli represented by (a) nuclear inductors derived directly from the genes, (b) early embryonic inductors or evocators and (c) hormonal stimulators which govern the developmental process of advanced stages. The following sections are devoted to the morphogenetic mechanisms: growth, differentiation, polarity, respiration and the metabolism of embryonic substances and structures. Here convincing demonstration is given of the fact that these processes, though normally geared together into a co-ordinated mechanism, can be disassociated from each other, not only conceptionally but also practically. This facilitates enormously the task of analyzing and resynthesising the complexities of living systems.

The host of these topics is lucidly dealt with, rendered readily accessible by the use of sub-headings, and commented with superior fair-mindedness, even in cases where the reliability of certain of the discussed findings might strain some reader's credulity. Notwithstanding his apology not to compete with existing textbooks on experimental embryology, the author surprises us with a compact source of information on many embryological subjects hitherto uncollected. Whenever possible these are considered from their chemical aspects and brought in relation to the dominant theme of organization. But it is not only the morphologist and biochemist who will derive a stimulative satisfaction from his book. With more

emphasis and authority than has been done before, Needham draws attention to the fact that the induction-competence principle, as elaborated mainly on amphibian embryos, has a far wider range of applicability than has commonly been realized, being perhaps of decisive importance in the determination of cancerous growth, of teratomata, of plant galls, of sex, and of a great number of hereditary and other malformations. It is to be hoped that especially medical students will avail themselves of the unparalleled opportunity here offered to get better acquainted with these chemical and morphogenetic links between processes of such wide phenomenological diversity.

The embryologist who is faced with this brilliant effort to picture large-scale biological phenomena as the outcome of chemicophysical reactions has to confess that his conception of the organizational forces which make for an integration of the part-processes into a synergetic whole are very vague. The concept of embryogenetic fields, proposed to fill the gap, suffers embarrassingly from the lack of a conclusive characterizability; its current meaning is as proteiform as there are authors speculating about it. It is not without compassion that we notice Needham's brave mind after having successfully fought the vitalistic demons falling a victim of the ambiguity of the "individuation field." Evidence is accumulating which makes it doubtful whether Waddington's concept of evocation, viewed as a separate feature within the induction process, does assist in clarifying the situation. According to recent findings, highly individuated neural inductions can be produced in explants without the application of an external evocator. This compels us to shift the responsibility for the specification of the induction more and more into the competent tissue itself, and to discredit the possibility of an interference of individuating host fields. We may add that such cases of "self-induction" also finally devalue the conclusiveness of the work of S. C. Shen which has been used by the Cambridge School as a last resort to defend their claims that the normally occurring neural inductors are of a sterol-like nature.

However, everybody familiar with the controversial flux of these problems will agree that such or similar criticisms count little in the evaluation of Needham's great piece of work. This will be a standard book for a long time to come. We owe much gratitude also to the Cambridge University Press for such a first-rate typographical achievement under the stress of war conditions. The text is permeated with beautiful photographic reproductions and an abundance of original schemes and diagrams. A glossary of the most commonly used embryological terms concludes the work.

J. HOLTFRETER, Montreal, Canada

The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes. By RICHARD ALAN MORTON of the Department of Chemistry in the University of Liverpool (England). Adam Hilger, Ltd., London, Second Edition, 1942. 226 pp. (Jarrell-Ash Company, Boston, Mass.).

The second edition of this survey book serves to keep the research worker up to date in the application of absorption spectra to the determination of the structure and concentration of important biological products. The use of absorption spectra as a tool to determine degree of unsaturation, location of important resonating linkages and the interrelation of multiple resonators has been of great importance in the study of natural products. The author has carefully sum-

marized the application to structural determination and analysis of (1) Steroids, (2) Provitamins and Vitamin A, (3) Vitamin E and Anti-oxidants, (4) Vitamin K, (5) Vitamin C and Vitamin P, (6) Vitamin B, (7) Purine and Pyrimidine Derivatives, and (8) Enzymes and Coenzymes.

The discussion on the first two fields (Steroids—Vitamin D) and (Vitamin A) constitutes about half of this survey volume, and in view of the extensive contributions of Dr. Morton to these fields these drafts are unusually authoritative. In those cases where the use of absorption spectra has been developed to include quantitative analysis, specific instructions are included as well as many special applications in the analysis of natural products.

The volume is well illustrated with spectrograms, absorption spectra curves, and equipment diagrams.

WALLACE R. BRODE, Columbus, Ohio

Mechanisms of Biological Oxidations. By DAVID E. GREEN, Senior Beit Memorial Research Fellow, Institute of Biochemistry, University of Cambridge. Cambridge University Press, London, 1941. 181 pp. Price \$2.75.

The present book is a reprinting of the first edition published in 1940. A few corrections, but no other changes, have been made and no new material has been added. Inasmuch as the previous printing was adequately reviewed at the time (*Science*, 93, 475 (1941); *Nature*, 148, 5 (1941)), this notice will be very brief.

The book is written from the viewpoint that since biological oxidations are catalyzed by enzymes, a discussion of the mechanisms of biological oxidations must be primarily a discussion of the enzymes involved. From the possible ways of classifying enzymes the one based on the chemical structure of the prosthetic groups is chosen. The result is a book divided into the following chapters: Introduction: General properties of oxidation enzymes; Fe porphyrin protein enzymes; Pyridinoprotein enzymes; Flavoprotein enzymes; Cu protein enzymes, with an addendum on a Zn protein enzyme; Thiaminoprotein enzymes; Cytochrome-reducing dehydrogenases; Unclassified oxidation enzymes; and Oxidations in organized systems.

The material is well presented in a brief and simple way and the book is the best single discussion of the group of enzymes covered that is available. However, considering the title of the book one wonders if the very desirable qualities of brevity and simplicity were not achieved by the effective but rather drastic procedure of leaving out everything that did not fit the simple pattern. For example, oxidation-reduction potentials are covered in one-half page and semiquinones receive only one very brief mention. While it is true that discussions of these subjects can be found in other places, it is also true that they have important and fundamental applications to the mechanisms of biological oxidations. Therefore, in the reviewer's opinion, the subject matter of the book, admirable though it is, does not adequately cover the field embraced by the title.

C. V. SMYTHE, Pittsburgh, Pa.

The Vitamin Content of Meat. By HARRY A. WAISMAN, Research Associate in Biochemistry in the University of Wisconsin, and C. A. ELVEHJEM, Professor of Biochemistry in the University of Wisconsin. Burgess Publishing Co., Minneapolis, Minnesota, 1941. IV + 210 pp. Price \$3.00.

The title of this monograph does not indicate by any means the range of material in the field of the vitamins which is treated therein. For all except a few of the more recently established vitamins, there is a discussion of the chemistry, physiology, pathology, therapeutics, methods of assay and the results of determinations on animal tissues. While the survey of the literature is brief, it is cogent and well organized. References to some recent important contributions are missing but this is understandable when the date of the copyright (1941) is considered in the light of the date of this review (April, 1943). The authors themselves recognize the fact that "most books on the vitamins are outdated the moment they are published."

The material in the early chapters has been taken from the literature; the authors have used only the pertinent data which in their estimation is the most reliable. Thus a survey of the physiological chemistry of vitamins A, D, E, K and C is given together with the content in animal tissues. The remainder of the book discusses in a similar way, the vitamins of the B-complex and in these chapters the authors have summarized the results of their own extensive studies. A detailed discussion of the preparation of the samples of tissue is given as well as a critical evaluation of the reliability of the methods of assay employed.

In the chapters dealing with the B-complex vitamins, the authors have evaluated the influence of cooking on the retention of the original vitamins of the samples of meat. Considerable criticism has been levelled at these results because of the failure to control the cooking temperatures. However, in a recent paper from the same laboratory, in which such control was maintained, data are presented which do not vary greatly from those in the monograph discussed in this review.

Throughout the monograph attention is directed to the importance of the nutritive value of meat and particularly to meat as a source of the vitamins. Both as a compendium of useful information and as a point of departure and stimulus for further work, this monograph represents a significant contribution to the current literature in the field of nutrition.

ARTHUR H. SMITH, Detroit, Mich.

Natural and Synthetic High Polymers. By K. H. MEYER, Professor of Organic Chemistry, University of Geneva, Switzerland. Interscience Publishers, Inc., New York, N. Y., 1942, XVIII + 679 pp. Price \$11.00.

This volume is the fourth in the series of monographs on High Polymers published under the editorship of Prof. H. Mark, Dr. E. O. Kraemer and Prof. G. S. Whitby. Together with *Physical Chemistry of High Polymers* by H. Mark (Vol. II of this series), the present work forms a translation of the second German edition of *Der Aufbau der Hochpolymeren Organischen Naturstoffe* by K. H. Meyer and H. Mark, the first edition of which was, unfortunately, never translated into English.

Considerable advance in both the ideas of the structure of high polymers and the theory of their solid state and solution properties has taken place since the first edition of this book appeared in 1930. In spite of this, however, considerable gaps in our knowledge exist and many topics continue to be the subject of controversy. In addition, new knowledge on these substances continues to accumulate at a high rate. All of this makes the author's task of presenting a "theoretical

discussion of the properties of these compounds" a very difficult one indeed. Professor Meyer, who has himself contributed outstandingly to the growth of the science of polymers, has succeeded in this assignment admirably. His volume enables those interested in high polymers to gain a breadth of view essential to constructive work in this field. To the biologist and even to the biochemist, many of the topics will seem off of the beaten path. Nevertheless those who will take time to study all sections of the book cannot help but be repaid in stimulating ideas, many of them applicable in their own work.

One of the troubles of any new branch of science is definition of special terms. At the present time high polymer chemistry is suffering from this terminology malady. Different terms are used for the same or almost the same thing and different phenomena are often described by the same term. In other cases old names continue to be employed which inadequately describe a structure or a phenomenon. Although an obvious effort has been made in the present book to establish the meaning of many of the terms used, there are many instances where the reader may become confused. For example, "crystallite" is used (see p. 407) to refer to a crystalline region in a polymer structure. This term was proposed by Staudinger and Signer in 1929 to refer to crystalline parts of high polymers in order to distinguish them from low molecular crystals. On page 251 the term "micell" (or micelle) as defined there would seem to have an identical meaning. In view of the variety of meanings which have been attached to the term micell, it might be simpler to refer to the submicroscopic crystalline regions as crystallites.

A book of such wide scope as the present volume on a rapidly growing and, in many ways, controversial subject cannot hope to be free of criticism and occasional error. In pointing out a few instances of this kind it is not intended to detract from the value of the work but rather to help acquaint the new reader with the intricacies of the subject.

On page 23 it is suggested that the limiting value of the reduced viscosity be called the "limiting viscosity" and that this quantity is identical with the limiting value of the intrinsic viscosity originally proposed by Kraemer. Actually Kraemer's intrinsic viscosity is identical with the present proposal and the need for a new term is therefore questionable. In this same section it is indicated that viscometric measurements "are unsuitable for the accurate determination of absolute values of molecular weight." Although the reviewer agrees that the method has been subject to much misapplication, nevertheless it is true that when properly applied, molecular weights can be determined accurately even at very high values as the recent work of Flory has shown. It is, however, necessary that the constants in the empirical viscosity equation are determined by a sound independent method such as end group titration or osmotic pressure, that linear molecules are present and that the polymer is either homogeneous or has a known molecular weight distribution.

On p. 182 it is stated that the fact that the pure polybutadiene shows an X-ray fiber diagram when stretched indicates that the chains are unbranched. Chains which are branched, however, may still give such a diagram provided the points of branching are not too closely spaced along the chain.

It has been customary in the X-ray work on polymers to attempt to work out

the dimensions of the unit cells and the arrangements of the molecule sections in them. A rather complete summary of the results on rubber, gutta percha, cellulose, and silk is given in the present volume. Although the importance of knowing the correct space group is evident, two things perhaps need to be emphasized perhaps more than they have. First, because of the scanty X-ray data furnished by high polymers and the fact that polycrystalline specimens must be worked with, it is difficult to arrive unambiguously at a space group. Secondly, the crystal structure per se is not always as significant for the properties of polymers as the amount and nature of crystalline-like order present. Thus (as the author points out on p. 644) Sponsler and Dore although they did not arrive at the correct space group for cellulose, nevertheless did point out facts on the basis of their X-ray results which had a profound effect on future thought on polymer structure.

A few comments might be inserted concerning the micellar versus the continuous structure theory of polymers. The author brings out (p. 251 and 653) in a very clear manner the differences between these two points of view and the arguments recently brought forward in support of the latter, stressing particularly the contributions of Frey-Wyssling made largely from the biological side. The growth of the continuous structure idea, as Professor Meyer points out, came about like most theories through a gradual modification of a host of related ideas on polymer structure, starting perhaps from the conceptions of Gerngross, Hermann and Abitz in 1930. One nevertheless feels that contributions of the English authors (Pierce, Neale and Astbury) in this connection might have been profitably included.

A number of tables giving technical properties of polymers important as plastics have been carried over from the German Edition. Inasmuch as many of these refer to German plastics and to German test results, they are not particularly valuable in the translation. Such technical tables are readily available in English and even these must be interpreted with care.

The author is frank in pointing out that the book is not offered as an exhaustive treatise although many subjects are treated in considerable detail. As he states in the preface the "chemist and biologist will not find in these pages a detailed practical guide to investigations in their special fields" but rather "a stimulus to new experimental and theoretical investigations." The volume covers all phases of polymer chemistry. Its contents may be roughly appreciated by the following topics: General Physical Chemistry of Polymer 125 pp., Inorganic Polymers 40 pp., Hydrocarbon Polymers 100 pp., Miscellaneous Organic Polymers 35 pp., Cellulose and Derivatives 165 pp., Starch 35 pp., Proteins 150 pp. All of these subjects are clearly treated and are supported by some 1500 references (counting duplicates). These form an excellent bibliography for supplementary reading and it is unfortunate that errors have crept into them.

Biochemists and biologists will find in the Chapter on Proteins many fresh points of view and the specialist in polymer chemistry will be gratified to find in one book subject-matter he previously has been forced to search for widely in the literature.

Finally the publishers are to be commended on the choice of paper, binding and general make-up of the volume. Typographical errors are few. The translator too deserves considerable commendation for a fine English version of this book.

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A Method for the Determination of Peroxidase Activity

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INTRODUCTION

Work on peroxidase in this laboratory has been somewhat hampered by lack of a satisfactory means of determining its activity. In 1918, Willstätter and Stoll (1) published a method which has been employed more frequently than any other. This calls for such large volumes of reagents that one-tenth quantities have usually been employed. We have found that with milkweed peroxidase the Willstätter and Stoll method gives results that are far too low, since milkweed peroxidase requires a higher peroxide concentration for its optimum activity than the kinds of peroxidase employed by Willstätter and his coworkers.

In this paper we describe a modification of the method of Willstätter and Stoll. We employ a small volume of digest, containing phosphate buffer and a relatively high concentration of hydrogen peroxide. After adding sulfuric acid to stop the enzyme action, the purpurogallin formed is extracted with ether, filtered, and the ethereal solution is read in a Fisher A. C. electrophotometer. The scale A reading is compared with a standard curve to obtain the mg. of purpurogallin. Peroxidase units are expressed as mg. of purpurogallin divided by 1000.

THE METHOD

Pipette 2 cc. of fresh 5 per cent pyrogallol, 2 cc. of 0.5 *M* phosphate buffer of pH 6.0, 15 cc. of water at 20°C. (or less water if a larger volume of enzyme is used) and 1 cc. of 1 per cent hydrogen peroxide into a 125 cc. Erlenmeyer flask. Place the flask in a thermostat bath at 20°C. and allow to come to temperature. Now add 1 cc. of properly diluted peroxidase, start a stopwatch and mix. After 5 minutes add rapidly 1 cc. of 2 *N* sulfuric acid, mix and extract the purpurogallin in a 150 cc. separatory funnel with 3 or 4 portions of ether. The total volume of ether

should be great enough to give a scale A reading of 10 to 55. Filter the ether extracts through a filter paper wet with ether into a graduated cylinder. Wash the container and then the filter with more ether so as not to lose any purpurogallin. Now mix the clear filtrate, take its volume and place some in a round electrophotometer tube of 23 mm. internal diameter. With a second tube containing only ether make a zero point reading, using the blue glass filter (4250 Å). Now read the unknown. Again make a zero point reading and again read the unknown. This redetermination of the zero point is necessary, since any considerable deflection of the galvanometer needle may change it.

TABLE I

Scale A readings with blue filter	Purpurogallin per 100 cc. ether <i>mg.</i>
5	0.38
10	0.69
15	1.00
20	1.30
25	1.65
30	2.05
35	2.50
40	3.00
45	3.50
50	4.00
55	4.63
60	5.40

To calculate the mg. of purpurogallin in the unknown solution use the figures in Table I, or a graph constructed from Table I. It must be taken into account that the mg. of purpurogallin in Table I are per 100 cc. of ether and that the mg. of purpurogallin in the unknown are in the volume of ether used. The peroxidase units are expressed as mg. of purpurogallin divided by 1000. These units are somewhat higher than the units of Willstätter and Stoll.

The scale A values given in Table I were obtained by making readings on ethereal solutions of purest purpurogallin of various dilutions, using the blue glass filter.

PREPARATION OF REAGENTS

The 0.5 *M* phosphate buffer of pH 6.0 is prepared by adding 840 cc. of 0.5 *M* KH_2PO_4 to 170 cc. of 0.5 *M* Na_2HPO_4 .

The 5 per cent pyrogallol is prepared every 2 days from Eastman Kodak Co. pyrogallol, m.p. 131–133°, and is kept in the ice chest.

SUMMARY

A modification of the method of Willstätter and Stoll for peroxidase activity is described. The purpurogallin formed by peroxidase and peroxide upon pyrogallol in the presence of buffer is extracted with ether, the filtered ethereal solution is read in a Fisher A. C. electrophotometer and the reading employed to obtain mg. of purpurogallin, using a standard curve. The peroxidase units are expressed as mg. of purpurogallin divided by 1000.

We wish to express our gratitude to the Rockefeller Foundation for a grant which made this work possible.

REFERENCE

1. WILLSTÄTTER, R., AND STOLL, A., *Ann. Chem.* **416**, 21 (1918).

A Comparison of Milkweed, Horseradish, and Turnip Peroxidases

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INTRODUCTION

Preliminary work in this laboratory indicates that the peroxidase of the milkweed (*Asclepias syriaca* L.) differs in several respects from the

TABLE I

Hydrogen Peroxide <i>per cent</i>	Milkweed Peroxidase Purpurogallin Formed <i>mg.</i>	Horseradish Peroxidase Purpurogallin Formed <i>mg.</i>
0.5	6.15	3.45
1.0	8.50	4.40
2.0	11.40	4.10
4.0	12.50	3.60
6.0	11.80	2 30

peroxidases of horseradish and turnip. Milkweed peroxidase requires a higher concentration of hydrogen peroxide for optimum activity than does horseradish peroxidase. This is shown in Table I with values obtained by the method of Willstätter and Stoll (1). The effect of peroxide concentration on milkweed, horseradish, and turnip peroxidases is shown graphically in Fig. 1.

EXPERIMENT AND DISCUSSIONS

Here a modification of the Willstätter and Stoll method was employed and the results are given in scale A readings made with an electrophotometer. It will be observed that both horseradish and turnip peroxidases have a sharply defined optimum hydrogen peroxide concentration, whereas milkweed peroxidase, which requires considerably more peroxide for its optimum, has a broad optimum zone and is not inactivated by

excess peroxide. Our results are probably not due to impurities, since both crude and highly purified milkweed peroxidase preparations have been found to give practically the same results. Our preparations were not entirely free from catalase, but we believe that the amounts of catalase present were far too small to have had any effect. Heating the peroxidase preparations to 70°C. or aerating, did not change the differences.

TABLE II

Order of addition of reagents

Kind of peroxidase	Order of addition of reagents		
	peroxide buffer water enzyme stood 5 minutes pyrogallol	pyrogallol buffer water enzyme stood 5 minutes peroxide	pyrogallol buffer peroxide water enzyme
	Peroxidase activity in Scale A readings		
Horseradish.....	56	61	63
Turnip.....	63	67	71
Milkweed.....	15	45	73

TABLE III

Scale A Readings

Temperature	Milkweed Peroxidase	Horseradish Peroxidase
50°C.	74.0	70.0
50°C.	73.0	55.0
60°C.	73.0	61.0
70°C.	69.0	58.0
80°C.	60.0	27.0
90°C.	42.0	2.0
98°C.	41.0	5.0

The order in which the reagents are added makes a difference in the activities of the peroxidases, as is shown in Table II. With milkweed peroxidase the difference is larger than with the other two peroxidases. With milkweed peroxidase the difference increases with time. This is shown both in Fig. 2, where the peroxide was added last, and also in Fig. 3, where the pyrogallol was added last. In all cases the time of digestion was 5 minutes.

When peroxidase and peroxide oxidize pyrogallol the initial speed of the reaction, as indicated by the initial slope of the curve (Fig. 4), is

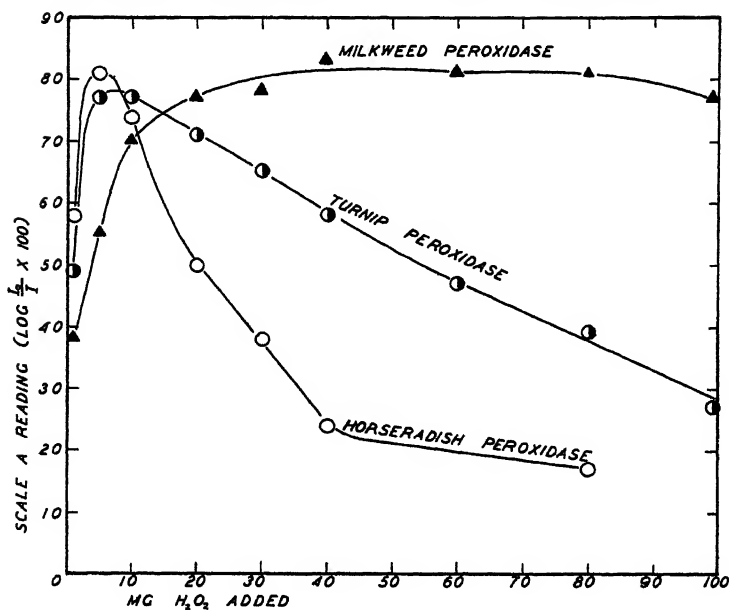
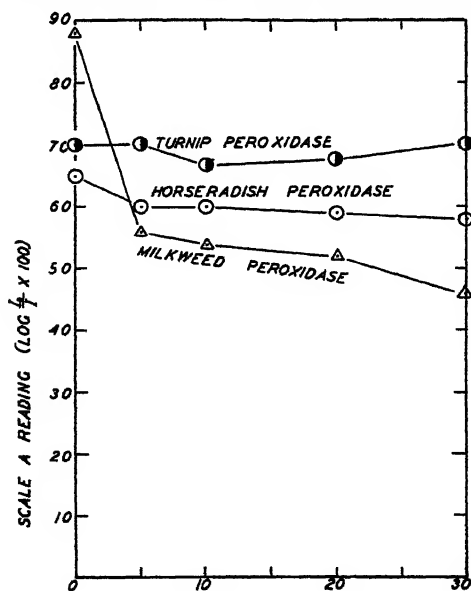


FIG. 1

The Effect of Concentration of Hydrogen Peroxide upon Peroxidases



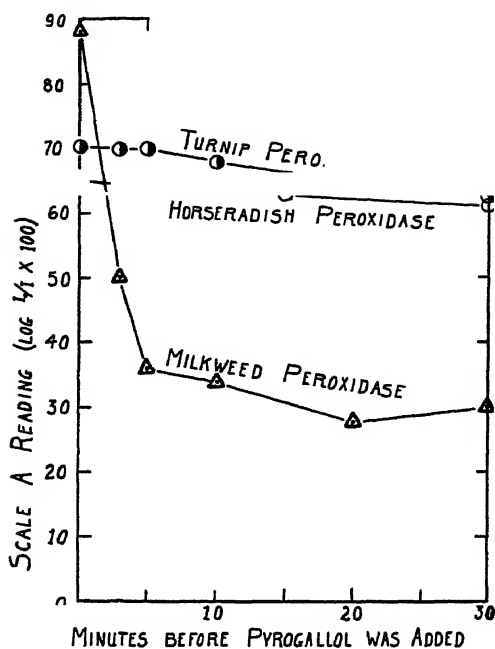


FIG. 3

The Effect of Standing with Hydrogen Peroxide upon Peroxidases

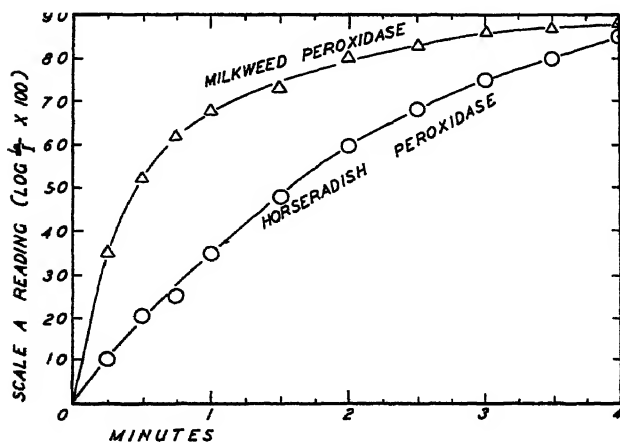


FIG. 4

The Initial Velocities of Milkweed and Horseradish Peroxidases

more rapid with milkweed peroxidase than with horseradish peroxidase. What this signifies we do not know. However, it is apparent that the two enzymes possess kinetic differences. Turnip peroxidase resembles horseradish peroxidase in this respect.

Milkweed peroxidase was found to be considerably more heat-resistant than horseradish peroxidase. This difference is shown in Table III. Here the time of heating was 4 minutes.

SUMMARY

Milkweed peroxidase has been shown to differ from horseradish and turnip peroxidases in requiring a higher concentration of hydrogen peroxide for optimum action and in not being inhibited by high concentrations of hydrogen peroxide. In addition, milkweed peroxidase is inactivated to a greater extent than the other two peroxidases when allowed to stand in contact with peroxide or pyrogallol. The initial speed of the reaction on pyrogallol is greater with milkweed peroxidase than with the other two peroxidases. Finally, milkweed peroxidase is more resistant to heat than the others.

We wish to express our gratitude to the Rockefeller Foundation for financial assistance.

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1. WILLSTATTER, R , AND STOLL, A., *Ann. Chem.* **416**, 21 (1918).

Microbiological Aspects of Streptothricin

I. Metabolism and Streptothricin Formation in Stationary and Submerged Cultures of *Actinomyces lavendulae**

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INTRODUCTION

Recent publications (1, 2, 3, 4) have attracted attention to the ability of certain actinomycetes to produce antibiotic substances. One such substance, streptothricin, produced by *Actinomyces lavendulae*, is unusual because it has antibacterial properties against Gram-negative bacteria in addition to Gram-positive organisms (3). This paper deals with the metabolism of and streptothricin formation by *A. lavendulae* and includes some studies on actinomycin formation by *A. antibioticus* (2). Some similar studies have been made by Waksman (5) employing the aerated culture technique previously described (6).

EXPERIMENTAL

Surface Growth and Streptothricin Formation

The marked variation among replicate cultures, which is more the rule than the exception with actinomycetes, made it a prerequisite to investigate conditions under which *A. lavendulae* grows more uniformly. The property of actinomycetes to develop slowly and to make relatively scant growth apparently accounts for the marked variations observed in the usual stationary liquid cultures or on solid substrates. Spores used as inoculum drop to the bottom of the liquid and made inconsequential growth, or a ring may develop at the surface-glass interphase. When

* This paper was presented at the joint meeting of the New Jersey and New York sections of the Society of American Bacteriologists in New York City, December 29, 1942. *J. Bact.* 45, 30 (1943).

surface pellicles do form, they are often incomplete and after a few days' growth gradually become wet and sink to the bottom, thereby arresting further growth and aerobic metabolic activity. Semi-solid agar (0.25 per cent) has been used (3) to provide some support for the pellicles of *A. lavendulae*.

Bottcher and Conn (7) have suggested for this purpose cotton saturated with nutrient medium. A medium consisting of tryptone 0.5 per cent, dextrose 1.0 per cent, NaCl 0.2 per cent, K_2HPO_4 0.2 per cent, was apportioned equally into large flat serum bottles to the depth of one and one-half inches. One flask was filled with absorbent cotton up to the surface of the liquid and about 100 mg. sterile talc added to another. A third received no treatment. The talc spreads over the surface and aids spores to remain floating. Yeast extract-glycerol medium was also tested with cotton. All were inoculated with *A. lavendulae* and incubated at 30°C. Only in the cotton and talc treatments did continuous dry pellicles remain on the surface. Pellicles in the untreated tryptone medium fell to the bottom of the containers on the third day. Streptothricin formation in these cultures is listed in Table I. Streptothricin was measured using *Bacillus subtilis* M in the cup assay previously described (8). For purposes of rough comparison, the expression of activity in terms of standard streptothricin units approximates the dilution causing inhibition of *Escherichia coli*. Both treatments in the tryptone medium gave good streptothricin activity, whereas the control gave none. Good growth and surface pellicle formation was obtained in the yeast extract-glycerol medium, but no streptothricin was produced.

The importance for streptothricin formation of securing abundant growth through good pellicle formation is further emphasized by a comparative study of a number of substrains isolated by plating out the stock culture of *A. lavendulae*. Growth and streptothricin formation were compared in tryptone medium in 250 ml. Erlenmeyer flasks containing 85 ml. medium. Marked differences between various strains were obtained (Table II). The ability to form more streptothricin did not appear to be distinctive for any special strains but corresponded closely with the total amount of growth made. In every case good growth could be correlated with the maintenance of a dry entire pellicle. When portions of a pellicle became wet, growth was retarded and streptothricin formation proportionally limited. However, when the better streptothricin producing isolates (nos. 1, 3, and 11, Table II) were rechecked, each in a number of replicate flasks, high and low strepto-

thricin titers were obtained among the replicates of each strain. As before, the more potent filtrates always had good surface growth and the low activity filtrates wet subsurface pellicles. Similarly, strains apparently poor in activity (nos. 6, 7, and 12), when rechecked in replicate,

TABLE I

Effect of Physical Support on Growth and Streptothricin Formation by A. lavendulae

Medium	Support	Pellicle Condition	Streptothricin			
			4 days	7 days units per ml.	8 days	11 days
Dextrose-tryptone	.	bottom	<10	<10	<10	<10
Dextrose-tryptone	talc	surface	20	50	40	30
Dextrose-tryptone	cotton	surface	20	75	50	<20
Yeast extract-glycerol	.. cotton	surface	<10	<10		<10

TABLE II

Growth and Streptothricin Formation by Substrains Isolated from a Stock Culture of A. lavendulae

Substrain	Streptothricin after		Growth*
	6 days	7 days units per ml.	
1	20	40	4
2	<10	15	1
3	30	40	4
4	<10	30	2
5	<10	<10	2
6	<10	<10	1
7	<10	<10	1
8	<10	15	2
9	30	<10	2
10	<10	15	2
11	30	40	4
12	<10	<10	1
stock	10	15	2

* Relative amount of growth; 4 indicates heavy growth and an entire surface pellicle.

yielded good and poor activities in direct correlation with the nature and amount. Thus, the variations in growth and streptothricin production encountered among replicates of identical treatments and strains of *A. lavendulae* tested under stationary conditions handicap greatly studies on the effects of imposed treatments. For example, repeated comparative testing would be required before any one strain could be selected as best from the 12 listed in Table II.

Streptothricin Formation in Submerged or Aerated Cultures

Cultivation of actinomycetes under conditions of aeration and agitation apparently has not been hitherto reported. Aeration either by shaking or by passing sterile air through the liquid leads to abundant growth occurring as a homogenous suspension of discrete colonies and mycelial fragments throughout the liquid. In favorable media growth occurs considerably more rapidly than under corresponding stationary surface conditions, and the extreme variation encountered in the latter instance is replaced by a surprising uniformity. Responses in growth and biochemical activities, as a result of imposed treatments, are obtained under homogenous physiological conditions and are not subject to the usual limiting factors of diffusion, concentration gradients, gas exchange, maintenance of pellicles, etc.

In the early stages of aerated cultures the cell material is not unlike a typical bacterial suspension, but by the third day the individual particles are distinguishable and are about the size of coarse grains of sand (Fig. 1, C and D). Two distinct types of mycelia are found in these submerged cultures (Fig. 2). One is a typical actinomycetous branching form with filaments of bacterial dimensions in width; the other is considerably thicker and fragments easily into small pieces, and resembles the aerial sporulating mycelium of colonies growing on solid media.

Streptothricin is formed in aerated cultures in amounts at least equivalent to stationary cultures and in appreciable shorter times. Table III compares streptothricin formation in different media. All values are averages of closely checking duplicates. Maximum activity in these experiments was reached in 5 to 7 days and dropped off rapidly thereafter. Supplements of neutralized corn steep liquor and soy bean meal were appreciably better for streptothricin formation than tryptone. In such media growth rate differences between aerated and stationary cultures are greater than in tryptone medium.

Fig. 3 compares various metabolic activities in aerated and in stationary cultures of *A. lavendulae* in dextrose-tryptone medium. The rate and time of maximum growth (dry weight) were parallel. The aerated cultures were always slightly ahead, but with certain other organic supplements they were considerably ahead. For example, in soy bean medium dry weights of cell material after 2 days were 5 and 153 mg. for surface and submerged cultures, respectively. Very little autolysis occurred after maximum growth even up to the tenth day. Over twice

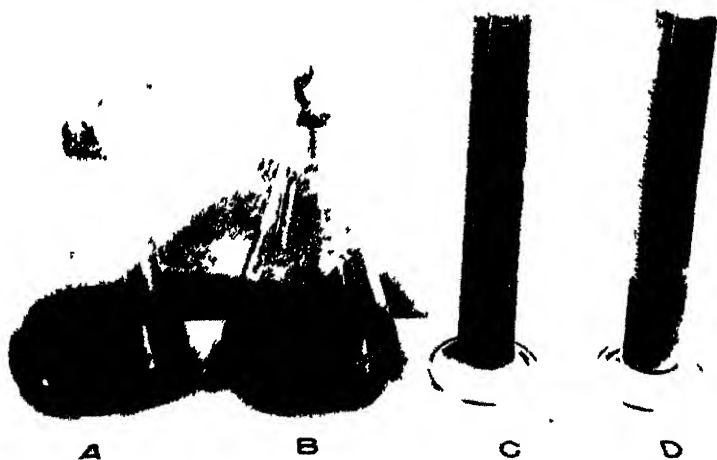


FIG. 1

- A. Stationary Culture With Surface Pellicle Growth of *A. lavendulae*
- B. Stationary Culture After Pellicle Has Dropped to the Bottom
- C. Aerated Culture Showing Uniformly Suspended Growth
- D. Aerated Culture Growth After Settling Out



FIG. 2

Mycelium of Aerated Culture of *A. lavendulae*

The thin hyphae correspond to vegetative mycelium and are of bacterial dimensions; the thick hyphae correspond to fragmenting or sporulating mycelium

Tryptone consumption was calculated from its nitrogen content after subtracting the total organic nitrogen (Kjeldahl) of the culture filtrates from that present originally. This calculation was in good agreement with wet combustion carbon determinations. There was little differ-

ence in total tryptone consumption, but in the aerated cultures it was considerably faster and paralleled dextrose consumption. This rapid utilization of organic nitrogen compounds in the presence of available carbohydrate is in keeping with a common observation on the metabolism of the actinomycetes, namely, that carbohydrates do not have to the same degree the protein sparing action they do with most saprophytic bacteria and fungi (9a). While much dextrose was still present, the organic nitrogen was attacked with liberation of ammonia in amounts considerably in excess of requirements for cell synthesis, again in accord with the well known deaminating properties of these organisms.

The pH changes are of special interest. Contrary to general belief (9b) acid formation from carbohydrate does occur, and to a degree sufficient to lower to pH 6.0 media buffered with K_2HPO_4 and in which appreciable ammonia accumulation has taken place. The drop in pH was sharp and of only 1 day's duration, after which it rose equally sharply into the alkaline range. The rise may be due to extra NH_3 formation or to actual oxidation of the acid. The short duration of acid production in both cultures may account for statements in the literature that actinomycetes do not form acids from carbohydrates. von Magnus (10) and von Plotho (11) have identified lactic acid in *Actinomyces* cultures, and changes in reaction toward the acid side were detected as early as 1919 (12).

Streptothricin formation reached a maximum coinciding with maximum growth in both cultures, the aerated cultures giving double the activity of the stationary. Its accumulation followed closely the development of the culture as indicated by dry weight and dextrose and tryptone consumption. Its maximum also coincided with the sharp drop in pH.

Acid Production by A. lavendulae

Acid formation in aerated cultures of *A. lavendulae* can be influenced by the carbohydrate concentration of the medium, especially in relation to the organic nitrogen (Table IV). In the absence of dextrose or with low concentrations of the carbohydrate, ammonia accumulation, as a result of the breakdown of tryptone, gradually made the medium alkaline. In the early days, acid formation was apparent in the 1 per cent dextrose set. The higher dextrose levels lowered the pH appreciably in the buffered medium, indicating formation of larger amounts of acid. In unbuffered media containing 2 per cent dextrose, pH levels as low

as 3.2 have been obtained in 2 days. This is in striking contrast to the usual observation that actinomycetes do not produce acid, are favored by alkaline reactions and will not develop at pH's less than 4.5 to 5.0, although some acid tolerant species are known (13).

The acid was identified as lactic acid. Filtrates from two day old aerated cultures in 85 ml. of 2 per cent dextrose-mineral media containing 0.5 per cent tryptone and 0.5 per cent glycine, respectively as nitrogen sources, were acidified and

TABLE IV
Acid Formation from Dextrose in Aerated Cultures of A. lavendulae

Dextrose concentration per cent	3 days	pH of medium* after 4 days	5 days	6 days
0	8.2	8.6	8.7	8.8
1	6.8	6.9	7.0	7.4
2	6.5	6.5	6.5	6.5
5	6.2	6.1	5.7	5.7

* Initial pH was 7.2.

TABLE V
Metabolic Changes and Efficiency of Carbon Utilization of A. lavendulae in Aerated Cultures

	Tryptone medium	Glycine medium
Mycelium weight.....	101 mg.	106 mg.
Dextrose disappeared.....	488 "	782 "
NH ₃ -N liberated.....	4 "	22 "
Nitrogen compounds deaminated.....	92 "	162 "
Lactic acid produced.....	126 "	58 "
Volatile acid as acetic.....	4 "	13 "
Conversion of dextrose to lactic acid.....	25.8 per cent	7.5 per cent
Conversion of glycine to acetic acid.....		10.3 " "
Efficiency of carbon utilization.....	24.8 " "	14.3 " "

extracted with ether in a continuous extractor. Aliquots of each culture were first reserved for residual sugar, NH₃, and cell nitrogen. Total acidity, after taking up the ether extracts in water, was determined by titrating while hot to include lactides. Qualitative tests with FeCl₃ and H₂SO₄-guaiacol indicated the presence of lactic acid. Quantitative lactic acid determinations were made on the neutralized ether extracts with a micro-modification of the Ulzer and Zeidel method. Steam distillations for volatile acids were also run. The analytical data are given in Table V.

In the tryptone medium the titratable acidity of the ether extract was practically wholly lactic acid with only a trace of volatile acid (acetic?).

In the glycine medium a significant proportion of the total acidity was due to volatile acid (acetic?). The lactic acid plus the volatile acid accounted for all the acidity in the ether extract. Undoubtedly the volatile acid was acetic formed by deamination of the glycine; the relatively large accumulation of ammonia indicates extensive deamination, and calculation from the Kjeldahl nitrogen in the cell material and ammonia N shows that 162 mg. of glycine had been deaminated. Volatile acidity calculated as acetic acid amounted to 10.3 per cent of the glycine decomposed. On the basis of sugar utilization there were 25.8 and 7.5 per cent conversions to lactic acid in the tryptone and glycine media, respectively. This high conversion is of particular interest because it took place under conditions of forced aeration. A similar effect has been noted with certain fungi of the genus *Rhizopus* (14). Lactic acid generally is believed to be a product of anaerobic metabolism in bacteria.

Efficiency of Carbon Utilization

Total dry weight of cell material synthesized was about the same in both cultures, but in the glycine treatment considerably more sugar was utilized to produce that growth. The efficiency of carbon utilization for cell synthesis by *A. lavendulae* is fairly high. The carbon and nitrogen contents of the cell material were 45 and 8.0 per cent, respectively. Carbon efficiencies calculated from the carbon of the dextrose and nitrogen sources destroyed but not accountable as soluble metabolic products (lactic and acetic acids), were 24.8 and 14.3 per cent for the two media, respectively. These values lie intermediate between average carbon efficiencies characteristic of the fungi and the bacteria. Taxonomically, actinomycetes are intermediate between the fungi and bacteria. Unless full account is given to the presence of soluble metabolic products, the efficiency of conversion values would be somewhat lower (19.9 and 13.2 per cent).

The higher efficiency of conversion in the presence of the complex organic nitrogen source tryptone as compared to glycine undoubtedly is due to the preformed or modified organic compounds which the organism assimilates directly and synthesizes into cell material or attacks more readily for energy, thus achieving a more efficient utilization of the available energy sources. Growth was considerably more rapid in the tryptone.

A comparison of efficiencies of carbon utilization in stationary and in

aerated cultures at different stages of development is given in Table VI. The stationary cultures are considerably more efficient at all stages of growth in converting substrates into cell material. Efficiencies of 35 per cent for the stationary cultures are unexpectedly high. This would approach the values for fungi which are among the most efficient micro-organisms in this respect. Bacteria generally are much less effective in conversion efficiency. In both cultures the over-all efficiency drops with progressive age. This probably can be explained by a more efficient assimilation of the carbon source and conversion into cell material during the active stages of growth. As the cultures grow older less synthesis takes place and additional consumption of the substrates consists of respiration ending in CO_2 , H_2O , and NH_3 . This continued

TABLE VI
Carbon Efficiency of A. lavendulae

Age	Carbon Efficiency		Sugar Consumed	
	Stationary per cent	Submerged per cent	Tryptone Consumed Stationary per cent	Submerged per cent
2	34.8	23.1	0.47	1.21
3	34.7	22.4	0.48	2.05
4	34.2	20.8	1.07	2.35
5		20.9		3.27
6		20.1		3.69
7		19.8		
8	26.0		1.60	3.12
10	24.4	17.7	1.62	2.42

utilization of substrate without concomitant conversion into cell material is the cause of the lowered over-all efficiency. Evidence for this is found in the ratio of sugar/tryptone consumption at the different ages. In both stationary and submerged cultures this ratio increases about 300 per cent in the later growth stages, indicating oxidation of more carbohydrate in relation to the tryptone utilized for cell synthesis. The 200 to 300 per cent higher ratios observed for submerged cultures accounts for their corresponding lower carbon efficiencies listed in Table VI. The submerged cultures, with more available oxygen, oxidized more carbohydrate per unit of tryptone utilized for synthesis of cell substance.

Studies on A. antibioticus

Fig. 4 presents the data on metabolic changes in tryptone medium in stationary and aerated cultures of *A. antibioticus*, a recently described

organism which produces two antibacterial substances, actinomycin A and B (2). Compared with *A. lavendulae*, growth in aerated cultures is considerably faster than under stationary conditions. The pH drop occurred only in the aerated cultures of this organism, no evidence of acid formation being apparent from the pH curves of the stationary culture. Again the pH drop is of brief duration and would be detected only by frequent analysis. The pH curves emphasize again that the ability to form acid from carbohydrates may be widespread among the actinomycetes but can be detected only if the proper conditions are

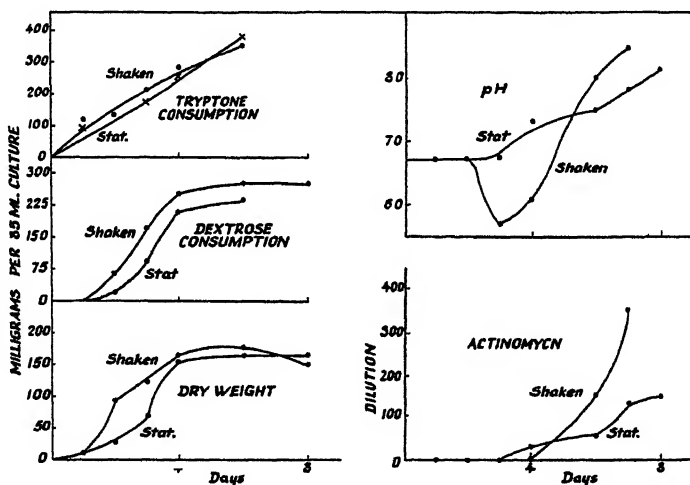


FIG. 4

Metabolism of *A. antibioticus* in Aerated and Stationary Cultures

established. An abundance of oxygen, as obtained in aerated cultures, apparently is necessary for acid formation. Production of actinomycin is considerably greater in the aerated cultures and was first detected on the fifth day, only after maximum growth had been reached. Actinomycin may therefore be a product of cell synthesis which is liberated rapidly by enzyme action or by autolysis after active growth has ceased.

Use of Aerated Culture Actinomycete Cell Material for Physiological and Biochemical Experiments

The uniformly suspended type of cell material obtained in aerated cultures of actinomycetes is excellently suited for experiments which

require homogenous physiological conditions. The individual particles of growth are small enough to permit pipetting and handling in much the same way as bacterial or yeast suspensions. Washed cell suspensions may be employed for biochemical studies, such as respiration, fermentation, methylene blue reduction tests, enzymes, etc. An example of a respiration experiment with washed cell material of *A. lavendulae* in a Warburg respirometer is shown in Fig. 5. The cell material is characterized by a fairly high endogenous autorespiration, but the cells oxidize dextrose and glycerol at an appreciable rate with 60 and 45 per cent of the theoretical oxygen uptake, respectively. The incomplete oxidation may

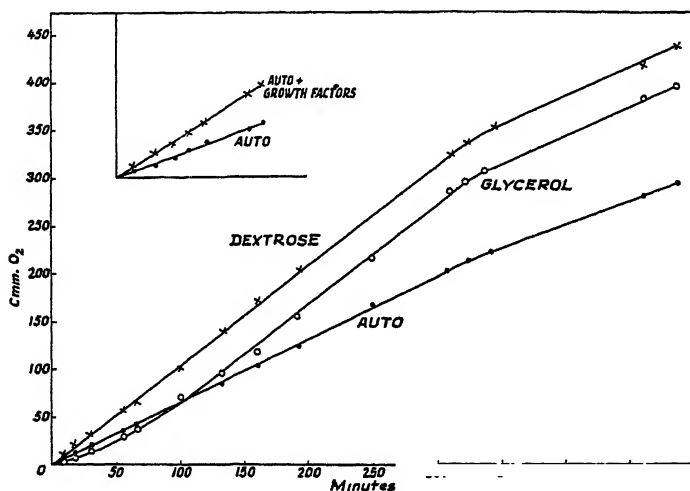


FIG. 5

Oxygen Consumption by Washed Suspensions of Aerated Cultures of *A. lavendulae* be due to oxidative assimilation or to the accumulation of lactic acid. Starvation for 1 to 2 days in phosphate buffer under aerated conditions reduces autorespiration appreciably, presumably by utilization of reserve cell materials. This process is accelerated in the presence of a mixture of small amounts of the B vitamins (inset, Fig. 5).

Deamination of Amino Acids

Washed aerated culture cell material of *A. lavendulae* was shaken 18 hours at 30°C. in the presence of 24 single amino acids. Five mg. of amino acid N in *M*/30 phosphate buffer at pH 6.8 were present in each flask. There was 7.6 mg. dry weight of cell material in each treatment.

Table VII shows the relative deamination of the amino acids as measured by ammonia formation. The majority of the amino acids are deaminated, with arginine and histidine being attacked most readily. β -Alanine is deaminated only about one-third as readily as α -alanine.

Streptothricin Assay Methods

A cup method for streptothricin determination using *Bacillus subtilis* M and based on the principle of the penicillin cup method (15) has already been described (8), and details will not be repeated here. A standard streptothricin preparation is employed and potencies are expressed in terms of streptothricin units. For reasons detailed else-

TABLE VII
Relative Deamination of Amino Acids by A. lavendulae

Degree of deamination							
good		fair		poor		none or slight	
Per cent of available N deaminated							
Arginine	27.3	Cysteine	3.4	<i>l</i> -Lysine	1.5	Norleucine	0
Histidine	22.0	Cystine	3.7	<i>dl</i> -Lysine	1.2	Isoleucine	0
Serine	10.8	<i>l</i> -Glutamic acid	4.0	β -Alanine	1.5	Phenylalanine	0.6
		<i>dl</i> -Glutamic acid	2.8	Tyrosine	1.4	Methionine	0.3
		<i>d</i> -Alanine	4.7	Proline	1.5	Leucine	0.3
		Glycine	3.1			Hydroxyproline	0.6
		Valine	2.2			Tryptophan	0.6
		Aspartic acid	2.8			Threonine	0.9

where (16), the cup assay is considered at the present time the most satisfactory method for streptothricin determination.

The importance of employing a streptothricin standard in assays is emphasized by the data from an assay experiment in which the plate dilution method was used without a standard. A stable preparation was assayed on seven different days and the inhibiting levels as expressed in terms of dilution of the preparation are listed in Table VIII. Such variations are not satisfactory for many types of quantitative chemical and biological studies.

In the cup method previously described, some question has arisen as to the values obtained on a given preparation when the one preparation is assayed at different concentration levels. In other words, does proportional zone formation occur independent of the concentration

level being assayed? Table IX shows that irrespective of the wide range of concentration level at which streptothricin is assayed by the

TABLE VIII

Daily Variation of a Streptothricin Preparation Assayed by the Plate Dilution Method

Inhibiting dilution¹ on different days

700-900

650

400-550

350-500

400

500-900

400

¹ Where two values are given, resistant colonies persisted in the lower dilution.

TABLE IX

Potency of Streptothricin Samples Assayed at Different Concentration Levels

Sample No. 1

Dilution of sample assayed	Standard curve reading corresponding to sample units	Streptothricin units in original sample units
4000	12	48,000
2000	27	54,000
1000	54	54,000
500	115	57,500
300	156	46,800
250	210	52,500

Average = 52,100 units

Maximum deviation = 11 per cent

Sample No. 2

4000	15	60,000
2000	37	74,000
1000	66	66,000

Average = 66,700 units

Maximum deviation = 11 per cent

cup method, the original sample is shown to contain the same number of streptothricin units. Maximum deviation in both cases was 11 per cent. The samples represented a highly purified preparation and a crude extract.

SUMMARY

The marked variability encountered in cultivation of actinomycetes in surface cultures is largely eliminated by cultivating these organisms

in a submerged condition with forced aeration, preferably with mechanical agitation. *A. lavendulae* and *A. antibioticus* have been studied in particular because of their ability to produce the antibacterial substances streptothricin and actinomycin, respectively. Growth and production of antibacterial substances generally are accelerated in aerated cultures as compared with stationary. Both species produce acid from sugar, and the process is favored by excessive aeration. The acid consists largely of lactic acid and amounts to as much as 25 per cent conversion on the basis of sugar consumed. Actinomycetes have a high order of efficiency of carbon utilization, converting 20 to 34 per cent of the carbon of the substrate consumed into cell material. Stationary cultures are more efficient than aerated cultures in this respect. Washed aerated culture actinomycete suspensions are suitable for general biochemical studies requiring homogenous physiological conditions, for example, Warburg respirometer experiments, methylene blue reduction, and other enzyme experiments. Relative rates of deamination of amino acids were determined. Features of the cup assay for streptothricin determination are discussed.

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The Fate of α -Estradiol and of Estriol Injected into a Human Male Subject*

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INTRODUCTION

In previous papers (1, 2) from these laboratories, data were presented on the isolation of estriol as a urinary metabolite of estrone injected into men. As a second metabolite α -estradiol was strongly indicated (2). Heard and Hoffman (3) have reported on the injection of α -estradiol into a human male subject; they isolated α -estradiol and estrone from the urines collected, but unfortunately discarded the fraction that might have contained estriol. We have repeated their experiment injecting a lesser quantity of α -estradiol, and in addition have conducted a similar recovery experiment after the injection of estriol.

METHODS

Control urine specimens were collected by one of us (J. S.) over a period of 12 days. Following the control collection period, a single intramuscular injection of 53.6 mg. of estriol triacetate in oil was made, and the post-injection urines were collected in 48-hour samples for an 8-day period. Five weeks later the same subject received a single intramuscular injection of 21.6 mg. of α -estradiol in oil, and collected three 48-hour post-injection specimens.

The urine hydrolysis and extraction employed was that described by Pearlman and Pincus (2). Following their methods three fractions were obtained: (a) a strong phenolic (estriol), (b) a weak phenolic ketonic (estrone), and (c) a weak phenolic non-ketonic (estradiol).

Each of the three phenolic fractions was assayed with spayed rats by our standard method (4). The determinations are ordinarily accurate to $\pm 15\%$ (4, 5).

RESULTS AND DISCUSSION

The data on the control specimens and the post-injection urines are presented in Table I. They demonstrate: (a) that a very large increase

* Aided by a grant from the National Research Council Committee for Problems of Sex.

in the activity of the strong phenolic (estriol) fraction occurs after the injection of estriol with no equivalent increase in the weak phenolic fractions; (b) that after the injection of α -estradiol the activities of all the phenolic fractions increase markedly; (c) that the increases of activity are greatest during the first 48 hours after estrogen administration, declining in the succeeding 48-hour periods.

Translating the data of Table I into micrograms equivalent of the estrogens presumably segregated into the three phenolic fractions, we

TABLE I

Estrogen Assays of Urinary Specimens Collected Before and After the Injection of Estriol and α -Estradiol

Specimen	Collection period, days	No. liters	Assay value, r. u. per 48 hrs.*		
			Weak phenolic non-ketonic (<i>Estradiol</i>)	Weak phenolic ketonic (<i>Estrone</i>)	Strong phenolic (<i>Estriol</i>)
Control.....	12	12.60	4.78	0.90	1.92
After estriol.....	1st 2	1.60	21.26	3.90(?)**	18,000.00
“.....	2nd 2	1.95	23.05	10.00(?)**	1,904.00
“.....	3rd 2	2.27	20.50	0.00(?)**	213.00
“.....	4th 2	2.20			27.80
After α -estradiol...	1st 2	1.83	1568.00	332.00	700.00
“.....	2nd 2	1.90	77.60	234.00	303.00
“.....	3rd 2	3.05	69.60	13.35	166.50
Total during 6 days after estriol.....	6	5.82	64.81	13.90(?)	20,117.00
Total during 6 days after α -estradiol.	6	6.78	1715.20	579.35	1,169.50

* By our assay method 1 r. u. = 0.125 micrograms α -estradiol, 1.0 microgram estrone and 1.0 microgram estriol.

** Insufficient activity for accurate assay.

present the data on the recoveries after α -estradiol injection in Fig. 1. Fig. 1 demonstrates: (a) that estriol is the principal urinary estrogen after α -estradiol injection, estrone is quantitatively intermediate, and α -estradiol is excreted in lowest amount; (b) that activity attributable to α -estradiol disappears most rapidly from the urine, that attributable to estriol least rapidly, estrone being intermediate.

In Table II, we compare our data on the urinary estrogen recoveries with those previously reported. The data of previous workers are not

completely comparable to ours. Heard and Hoffman (3) report recoveries of crystalline estrogen after the injection into a single subject of

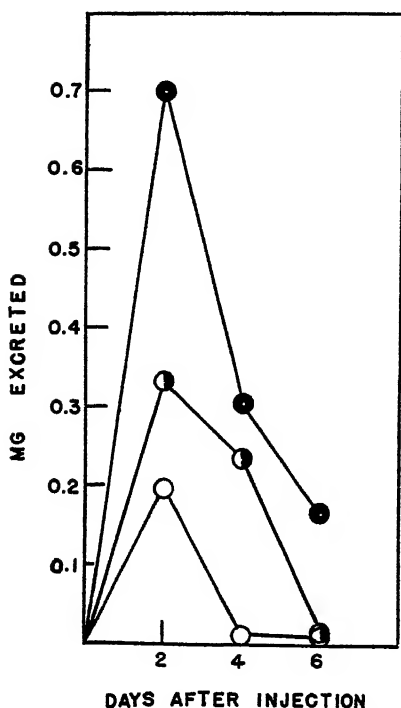


FIG. 1.

The Urinary Excretion of Estrogen After the Injection of α -Estradiol

The activity of the three principal phenolic fractions is expressed in mg. equivalent of the presumed estrogens segregated into the respective fractions. Open circles: α -estradiol (weak phenolic non-ketonic); half-circles: estrone (weak phenolic ketonic); closed circles: estriol (strong phenolic). See Table I

of injected estrogen is unaccounted for by urinary recoveries after the injection of estrone and α -estradiol.

We believe that these data establish for the human male the course of

α -estradiol over a period of eight days with urine collection including the ten days following the last injection (*i.e.* 18 days collection in all). Furthermore, their method of urine hydrolysis was not the same as ours. The injection and hydrolysis and extraction methods of Pearlman and Pincus (2) were followed by us, but their data are assays based on four days post-injection collection from 7 men who received aliquots of the estrone (as the acetate). Nonetheless from these data certain deductions are permissible. These appear to us to be: (a) estriol is not converted to estrone or estradiol *in vivo* (the slight increase in estradiol assay in the estriol injection series, we attribute to the partitioning of small amounts of estriol into the non-ketonic weak phenolic fraction); (b) estrone and α -estradiol are intra-convertible, and the injection of both leads to the urinary excretion of estriol also; (c) unless there are potentiators of the action of estriol in the strong phenolic fraction, the data indicate that approximately half of the injected estriol is excreted into the urine; (d) approximately 90%

estrogen conversion previously postulated for the human female (6, 7, 8) and for most mammals (9, 10, 11) subjected to estrogen injection studies. The rather large quantitative recovery of estriol noted in our data has been paralleled in experiments with women by Smith, Smith, and Schiller (8), and these authors have noted no indication of estriol conversion to the other two native estrogens. Failure of conversion of estriol to recognizable metabolites has been recorded by Pincus and Zahl (9) and Doisy (10). The apparent large estriol recovery may be due in part to the presence of inactive materials in the strong phenolic fraction that

TABLE II

The Recoveries of Estrogen in the Urine of Men Injected With α -Estradiol, Estrone, and Estriol

Estrogen injected	Amount injected	Recovered in the urine as						Source of data
		α -Estra- diol		Estrone		Estriol		
		as % of in- jected		as % of in- jected		as % of in- jected		
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
α -Estra- diol†.	250.00	9.80	3.92	16.20	6.48	†	*	Heard and Hoffman (3)
α -Estra- diol†...	21.60	0.21	0.99	0.58	2.68	1.17	5.42	This paper
Estrone‡	911.40	3.53	0.39	23.20	2.55	28.50	3.13	Pearlman and Pin- cus (2)
Estriol‡...	37.34	0.08	0.02	0.14(?)	0.03(?)	20.12	56.57	This paper

* Not determined.

† Injected as the free estrogen.

‡ Injected as the acetates, amount injected calculated as free estrogen.

enhance the activity of the native estrogen. This is suggested by the data of Pearlman and Pincus (2) who obtained 10 mg. of crude crystalline material from a strong phenolic fraction having an activity equivalent to 28.5 mg. estriol. Pincus and Pearlman (4) found indications of such material in the urines of cancerous persons and of non-cancerous men. On the other hand these authors (4) and Smith and Smith (7) find that normal women excrete inhibiting, rather than potentiating material. The need for further investigation is indicated, but the scheme of conversion originally proposed by Pincus and Zahl (9):



still holds as a tenable, and the most likely, hypothesis. The possibility that estriol may be derived directly from estradiol rather than secondarily from estrone is not excluded. Against this possibility, however, is the rapid rate of disappearance from the urine of α -estradiol after its injection, and the continuing excretion of estrone and estriol (Fig. 1). Otherwise we must infer a differential storage of estrogen in the body, but the data of Cantarow *et al.* (12) on the storage of estrogen in the bile give no such indication. The suggestion of Heller (13) that estrone is converted to α -estradiol by certain tissues *in vitro* (see also Twombly and Taylor (14)) is not controverted by the data on urinary excretion since in the latter studies the over-all effect of changes *in vivo* is measured. A direct determination of the metabolizing mechanisms will undoubtedly give further clues to the conversion processes.

SUMMARY

After the intramuscular injection into a human male subject of estriol diacetate, 56.6% of the activity was recovered in the strong phenolic (estriol) fraction of the urine collected for six days after injection. After a single injection of α -estradiol increased activity accountable to estradiol (1.0%), estrone (2.7%) and estriol (5.4%) was obtained. The significance of these findings for the general problem of estrogen metabolism *in vivo* is briefly discussed.

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The Nutrition of *Proteus Morganii*

Amino Acid and Growth Factor Requirements

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INTRODUCTION

In a preliminary report on the nutritional requirements of *Proteus morganii* it was demonstrated that nicotinic acid and pantothenic acid are essential growth substances for this organism (Pelczar and Porter, 1940). Thirty-seven strains of the species were cultivated in a chemically-defined medium, consisting of several amino acids, glucose, inorganic salts, and the above growth factors. It was observed, however, that this medium supported only limited growth on continued subculture. Quantitative measurements of growth in the chemically-defined medium and in a more complex substrate such as glucose meat-infusion broth, showed that the latter was greatly superior from the standpoint of production of bacterial cells in a known volume of medium.

The experiments described in this report are the detailed results on the amino acid and growth factor requirements of *Proteus morganii*. Also an attempt has been made to construct a chemically-defined medium for this species which would compare, in terms of total growth, to an ordinary laboratory medium such as glucose meat-infusion broth.

TECHNIQUE

Bacteriological Methods and Cultures

The strains of *Proteus morganii* employed were typical in their cultural and physiological characteristics and were maintained on nutrient agar slants. The

* We wish to thank Merck and Co., Inc., for generously supplying most of the vitamins and several of the amino acids employed in this study; also Dr. D. Wright Wilson, Univ. of Pennsylvania, for the supply of octopine used.

inoculum for the various media investigated was prepared as follows: A trace of growth was removed by means of a platinum wire from a 24-hour agar slant culture and placed into 10 ml. of the basal medium (described below). This was incubated for 24 hours, after which one drop (approximately 0.05 ml.) was transferred to 10 ml. of similar medium. After a 24-hour incubation period this culture was employed as the inoculum. One drop (approximately 0.05 ml.) from a 1.0 ml. pipette was used throughout for the inoculation of 10 ml. of media.

Quantitative measurements of growth were made by determining the turbidity of a liquid culture with a Klett-Summerson photoelectric colorimeter. The instrument was adjusted to zero against the uninoculated medium, and the turbidity readings were taken directly from the scale. In one instance (Table I) quantitative measurements were also made by determining the total amount of bacterial nitrogen in a given volume of liquid culture, following the procedure described in another report (Porter and Pelczar, 1941).

The basal medium employed had the following composition:

NH ₄ Cl.....	1.0 g.
(NH ₄) ₂ SO ₄	1.0 "
NaCl.....	1.0 "
KH ₂ PO ₄	1.0 "
K ₂ HPO ₄	1.0 "
MgSO ₄	0.1 "

Distilled water to 950 ml.

pH adjusted to 7.2-7.4 with N/NaOH

This medium was tubed in 9.5 ml. amounts, autoclaved and then fortified with the following:

Glucose (0.1 ml. of sterile stock solution) to give final concentration of 0.5 per cent;

Cystine (0.1 ml. of sterile stock solution) to give final concentration of *M*/10,000;

Ca-pantothenate (0.1 ml. sterile stock solution) to give final concentration of 1.0 microgram per ml.;

Nicotinamide (0.1 ml. of sterile stock solution) to give final concentration of 1.0 microgram per ml.;

Sterile distilled water to make 10 ml.

The amino acids and growth factors were prepared individually in solutions, sterilized by sintered glass filtration, and added to the basal medium. The amount of distilled water was reduced when substances other than those listed above were added so that all of the media could be adjusted to a final volume of 10 ml.

EXPERIMENTAL

Quantitative Comparison of Growth in Basal Chemically-defined Medium and Glucose Meat-Infusion Broth

The inadequacy of the chemically-defined medium from the standpoint of supporting maximum growth of *Proteus morganii* in a given vol-

ume of medium is evident from the results presented in Table I. In each instance the mass of growth in the infusion medium is practically double that which took place in the same volume of "synthetic" medium whether the estimation was made by bacterial nitrogen determinations or by turbidity measurements. However, it was possible to fortify the basal chemically-defined medium with trace amounts of extracts of several animal tissues, and obtain a quantity of growth corresponding to the yield from glucose meat-infusion broth (Pelczar and Porter, 1941). This indicates that for optimum growth of *Proteus morganii* some addi-

TABLE I

Comparison of Quantity of Growth Resulting from Cultivation of Proteus morganii in Synthetic and Non-Synthetic Media

Medium	Culture Number and Type of Determination*							
	6		14		18		24	
	I*	II*	I	II	I	II	I	II
Glucose (0.5%) meat-infusion broth	2.16†	78†	2.30	90	2.11	95	2.10	87
	1.98†	87†	2.14	88		90	2.19	96
Chemically-defined medium‡	1.16	46	1.13	42	0.98	40	1.29	51
	1.12	49	0.91	44	1.09	40	1.18	52

* I, Quantity of growth in terms of bacterial nitrogen, mg./50 ml. media.

II, Quantity of growth as measured by turbidity, using a Klett-Summerson colorimeter. Instrument set at zero with blank media.

† Determinations on different days.

‡ Basal medium in text plus 18 amino acids (Group A, Table III).

tional factor (or factors) must be present in a medium of known chemical composition.

The following data are the results of an attempt to increase growth in the basal medium with additions of known chemical entities, namely, vitamins, amino acids and their derivatives, and other substances which have been reported as having a stimulating effect upon bacterial growth.

Determination of the Effect of Amino Acids and their Derivatives

Nitrogen and Sulfur Requirements. Early in the investigation of the nutritional requirements of *Proteus morganii* it was discovered that inorganic nitrogen salts would not serve as the sole source of nitrogen; consequently, a mixture of 18 amino acids was incorporated in the

medium (Group A, Table III). Later, by a process of elimination it was found that the only type of amino acid which was essential for the growth of *Proteus morganii* was one containing sulfur; *i.e.*, cystine or methionine. The effect of these two amino acids on growth is presented in Table II. From these results it is apparent that the addition of either cystine or methionine to the "synthetic" medium is necessary before the organisms can initiate growth. Of the two amino acids, cystine permits the best

TABLE II
Determination of Essential Amino Acids for Proteus morganii. The Indispensability of Cystine and its Replacement by Methionine

Medium	Culture Number				
	1	10	15	21	37
	Nephelometer readings*				
Basal medium without amino acids.....	0	1	3	2	1
Basal medium with all of the amino acids in Group A, Table III, except cystine and methionine.....	2	3	1	4	3
Basal medium containing only cystine (<i>M</i> /10,000).....	51	48	51	59	50
Basal medium containing only methionine:					
<i>M</i> /2,000.....	40	31	42	37	33
<i>M</i> /4,000.....	22	25	37	30	35
<i>M</i> /10,000.....	18	22	30	21	30
Basal medium plus cystine (<i>M</i> /10,000) and methionine (<i>M</i> /4,000).....	48	39	50	47	49

* Average results of repeated determinations.

growth. Increasing or decreasing the concentration of methionine above that ordinarily employed (*M*/4,000) does not seem to make it as efficient a source of organic sulfur as cystine.

Apparently, only sulfur-containing amino acids are essential for *Proteus morganii* to initiate growth. The original medium contained 18 amino acids (Group A, Table III) but when these were omitted individually, no significant change in growth was observed since cystine or methionine was always present.

TABLE III

Amino Acids and Related Compounds Used as a Supplement to the Basal Medium

Group	Amino Acids Employed	Final concentration of each amino acid per ml. of medium
A	<i>dl</i> -alanine.	M/1,500
	<i>d</i> -arginine, HCl.	M/4,000
	<i>l</i> -aspartic acid.	M/1,500
	<i>l</i> -cystine.	M/10,000
	glycine, A.P.	M/1,500
	<i>dl</i> -glutamic acid.	M/1,500
	<i>l</i> -histidine HCl.	M/4,000
	<i>l</i> -hydroxyproline.	M/1,500
	<i>dl</i> -leucine.	M/1,500
	<i>dl</i> -lysine, HCl.	M/4,000
	<i>dl</i> -methionine.	M/4,000
	<i>l</i> -proline.	M/1,500
	<i>dl</i> -phenylalanine.	M/4,000
	<i>dl</i> -serine.	M/1,500
	<i>dl</i> -threonine.	M/1,500
	<i>l</i> -tryptophan.	M/20,000
	<i>l</i> -tyrosine.	M/4,000
	<i>dl</i> -valine.	M/1,500
B	β -alanine.	M/1,500
	<i>dl</i> - α -amino- <i>n</i> -butyric acid.	M/1,500
	<i>dl</i> - β -amino- <i>n</i> -butyric acid.	M/1,500
	α -amino- γ -phenyl- <i>n</i> -butyric acid.	M/2,000
	ϵ -amino- <i>n</i> -caproic acid.	M/1,500
	<i>dl</i> - α -amino- <i>n</i> -caprylic acid.	M/1,500
	β -amino- <i>n</i> -valeric acid.	M/1,500
	<i>dl</i> - γ -amino- <i>n</i> -valeric acid.	M/1,500
	<i>dl</i> - δ -amino- <i>n</i> -valeric acid.	M/1,500
C	betaine, HCl.	M/1,500
	<i>dl</i> -asparagine.	M/1,500
	<i>dl</i> -citrulline.	M/4,000
	hippuric acid, C.P.	M/1,500
	iodogorgoic acid.	M/4,000
	<i>dl</i> -isoleucine.	M/1,500
	<i>dl</i> -norleucine.	M/1,500
	<i>dl</i> -norvaline.	M/1,500
D	<i>d</i> -ornithine, 2HCl.	M/1,500
	octopine.	M/1,500
	sarcosine.	M/1,500
	taurine.	M/1,500
	allothreonine.	M/1,500

The Effect of Several Amino Acids and their Derivatives upon the Growth of Proteus morganii. It has been observed repeatedly in studies on the nutrition of this species that aqueous extracts of animal materials contain some substance (or substances) which, when added in a small amount to the basal medium, brings about a marked increase in both the growth rate and total mass of growth of *Proteus morganii*. Reasoning that the stimulative factor (or factors) might be one of the uncommon, but commercially available, amino acids, several compounds were tested to determine their activity; these compounds are listed in Table III. Individual stock solutions were prepared and sterilized by passage through sterile sintered glass filters. The concentration of the individual compounds in stock solution was such that when 0.1 ml. was incorporated in 10 ml. of the basal medium, its final concentration in the medium would correspond to the figures given in Table III. The basal medium was fortified individually and in combinations with each of the groups (A, B, C, and D) of amino acids listed in Table III. No significant increase in growth occurred after these additions had been made. Instead, when either Group C or Group D was added separately to the medium, a marked decrease in growth resulted. Since it has been observed by Gladstone (1939) that various interrelationships exist between combinations of amino acids in so far as stimulation or inhibition of growth of *Bacillus anthracis* is concerned, it is probable that some similar interrelationship exists for *Proteus morganii*. Thus, it was decided to investigate the influence of the compounds in Groups C and D by adding them individually to the basal medium. The results showed that norvaline, norleucine, and allothreonine in the concentrations employed exhibited a marked inhibition of growth; in fact, in some cases growth was delayed for 48 to 96 hours, or was inhibited entirely. This inhibitory effect, however, was partially or completely neutralized when each of these compounds was present in the medium with other amino acids; for example, when present in a medium containing the 18 amino acids listed in Group A, Table III. The interrelationship between various amino acids in the nutrition of *Proteus morganii* is being investigated and will be reported later.

The Effect of Several Vitamins, Purines and Pyrimidines, and other Bacterial Growth Factor Substances upon the Growth of Proteus morganii in a Chemically-Defined Medium

Several substances known to be required for certain bacteria to grow in "synthetic" media were tested for any stimulation they might

possess for the growth of *Proteus morganii*. The compounds, listed in Table IV, were all sterilized by passage through sintered glass filters

TABLE IV

The Influence of Several Vitamins and Related Substances upon the Growth of Proteus morganii in a Chemically-Defined Medium

Media		Culture number				
		1	10	15	21	37
		Nephelometer readings				
Basal medium		50-54*	43-46	57-59	58-62	55-55
Basal medium +	Concentration μg./ml.					
thiamine.....	10					
riboflavin.....	10					
pyridoxin.....	10					
choline, HCl.....	50					
biotin.....	1	52-56	39-44	58-59	57-58	56-64
folic acid.....	1					
para-amino benzoic acid.....	10					
inositol.....	100					
β-alanine.....	50					
pimelic acid.....	50					
glutamine.....	10					
d-glucosamine, HCl.....	50					
Basal medium +						
uracil.....	10					
cytosine.....	10					
adenine.....	10					
thymine.....	10	54-56	44-51	60-61	61-65	60-64
guanine.....	10					
xanthine.....	10					
barbituric acid.....	10					
adenylic acid.....	10					

* Duplicate determinations.

and incorporated in the medium in amounts as tabulated in the same table.

None of the vitamin B-group gave any increase in growth beyond that which was observed in the basal medium (containing pantothenic acid

and nicotinamide); nor did the addition of glutamine, *d*-glucosamine, HCl, indole-3-acetic acid, glutathione, or ascorbic acid cause any increase on growth. Although not shown in the results of Table IV, macroscopic observation of the culture tubes during intervals of the 24-hour incubation period showed that bacterial growth (as measured by turbidity measurements) occurred much more rapidly in those tubes which contained the purine and pyrimidine bases. However, no significant increase in total growth was observed after incubating for 24 hours.

DISCUSSION

Proteus morganii can be cultivated on continuous subculture in a chemically-defined medium containing inorganic salts, glucose, cystine, pantothenic acid, and nicotinamide. However, in this medium only moderate growth occurs, being approximately half that which is obtained in the same volume of glucose meat-infusion broth. Since the addition of small amounts of animal tissue hydrolyzates or extracts to the basal chemically-defined medium resulted in marked stimulation of growth, various media were prepared and fortified with several known amino acids and growth factors to determine if the stimulant was one of these substances. Since none of these compounds tested produced any great stimulative effect on the organism in chemically-defined media, some unknown factor appears to be required by *Proteus morganii*. Work on the nature of this factor is now underway.

Cystine is an essential amino acid from the standpoint that it must be supplied to the organisms before growth is initiated. A concentration of $M/10,000$ of cystine in the medium appears to be optimum. Methionine may replace cystine, but is not so effective. Concentrations of $M/2,000$ to $M/10,000$ methionine in the chemically-defined medium result in poorer growth of *Proteus morganii* than when $M/10,000$ cystine is present. The presence of both cystine and methionine in the basal medium is of no advantage to the organism. Cystine has been repeatedly identified as a growth requirement for several other bacteria when they are cultivated in "synthetic" media, *e.g.*, lactic acid bacteria (Wood, Geiger, and Werkman, 1940), *Corynebacterium diphtheriae* (Mueller, 1935), *Clostridium sporogenes* (Fildes and Richardson, 1935), and *Staphylococcus aureus* (Fildes and Richardson, 1937).

We have previously noted that the nutritional difference between *Proteus morganii* and the other species of the genus *Proteus* is that the

former requires panthothenic acid and nicotinic acid (or amide), while the latter require only nicotinic acid for growth in simplified media. An additional point of variance has been observed here since *Proteus morganii* requires cystine, while practically all other species of *Proteus* require no added organic sulfur compound for growth in simple media.

Of some 39 additional amino acids and derivatives which were employed to fortify the basal medium none brought about any significant stimulation of growth. There was evidence of inhibition of growth by three amino acids, namely, norvaline, norleucine, and allothreonine. Norvaline ($M/1,500$) and norleucine ($M/1,500$) caused a complete inhibition of growth, while allothreonine ($M/1,500$) reduced growth to just a trace in 24 hours. It is interesting to note that when these substances were incorporated in media along with several other amino acids the inhibitory effect was very much reduced; the toxic manifestation of norvaline, norleucine, and allothreonine is, therefore, blocked in some way by the presence of other compounds in the groups. None of the better known growth factors of the vitamin B-complex, except nicotinamide and pantothenic acid, has any stimulative effect on the growth of *Proteus morganii*.

Recent experiments have disclosed the significance of purine and pyrimidine compounds in bacterial nutrition. Snell and Mitchell (1941) have shown that each of the purine and pyrimidine bases which occur naturally in nucleic acids may be limiting factors for growth of several strains of lactic acid bacteria. Previous to this, Richardson (1936) demonstrated that uracil was essential for the anaerobic growth of *Staphylococcus aureus*, Möller (1939) showed that adenine was required for the growth of *Streptobacterium plantarum*, and Pappenheimer and Hottle (1940) discovered that adenine was necessary for the growth of a strain of Group A hemolytic streptococcus. For the latter organism, adenine could be replaced by hypoxanthine, guanine, xanthine, guanylic acid, or adenylic acid. For *Proteus morganii* the purine and pyrimidine compounds appear to stimulate the growth rate when added to a synthetic medium. However, no increase in the final total amount of growth was noted.

SUMMARY

The amount of growth of *Proteus morganii* in a given volume of chemically-defined medium is approximately one-half that occurring in the same volume of glucose meat-infusion broth. The addition of some 37

amino acids and several bacterial growth factors to a chemically-defined substrate produced no significant increase in growth, although the addition of small amounts of crude animal or plant extracts, or hydrolyzed casein do stimulate growth. The nature of the substance (or substances) needed for optimum growth of this organism in media of known composition is being investigated further.

Cystine is an essential amino acid for the growth of *Proteus morganii*. It may be replaced by methionine but not so effectively.

Norvaline (*M*/1,500), norleucine (*M*/1,500), and allothreonine (*M*/1,500) when incorporated separately into the chemically-defined basal medium were distinctly inhibitory to the growth of *Proteus morganii*. However, when other amino acids are also present this inhibitory action is neutralized.

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Determining Riboflavin in Dried Milk Products

III. A Comparison of the Methods of Assay¹

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INTRODUCTION

Several years ago, one of us developed a photometric procedure for measuring riboflavin in dried milk products (1). The method has proven satisfactory when applied to these particular substances and has been used for the assay of hundreds of samples (2). However, since it could not be applied to numerous other materials, we occasionally found it necessary to resort to biological assays. One purpose in developing the chemical method, aside from the obvious one of speed, was the avoidance of errors frequently encountered in biological assays. Therefore, it appeared necessary to check the biological methods against the chemical method before too much reliance could be placed on the former.

In a preliminary report, it was shown that, with certain dairy products, all biological methods which were tried gave too high results (3). At the same time, other workers found that good agreement was obtained between fluorometric methods and two different biological assays (4). Publication of our results has been withheld until further data was accumulated in an attempt to explain the discrepancies observed. While we are still unable to give a complete explanation of these results, repeated trials have revealed interesting information regarding biological assays as well as further knowledge concerning the nutritive value of dairy products. .

METHODS

During the past few years, numerous modifications and improvements have been made in the original (5) Bourquin-Sherman technique of as-

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saying for riboflavin. As a result, we have changed the ration and level of assay from time to time, but since the general procedure is well known, no attempt will be made to describe this assay in detail. Likewise, we will omit any description of the photometric procedure which was given in detail in the first paper of this series (1).

Microbiological assays were made according to the procedure of Snell and Strong (6), except where modifications are noted. In preparing extracts for these tests, the solutions used in the photometric tests were diluted to an appropriate

TABLE I
Test of Microbiological Assay for Riboflavin

Riboflavin in micrograms	Acetone in ml.	Dried whey extract equivalent mg.	Acid in ml.	Riboflavin in micrograms per gram
0	0		2.1	
0.1	0		6.1	
0.1	0.010		6.1	
0.1	0.025		6.0	
0.1	0.040		6.3	
0.1	0.100		6.0	
0	0		1.6	
2.0	0		9.6	
2.0	0.01		9.5	
2.0	0.05		9.6	
2.0	0.10		9.6	
0	0		1.2	
0.1	0		2.8	
0	0	2.5	1.9	22
0	0	5.0	2.9	21

volume with distilled water. Thus, if any losses are encountered due to the extraction procedure, they will be common to these two methods of assay. Although a large number of determinations have been made by this procedure at the present time, the method was quite new at the beginning of this study. Therefore, it appeared wise to conduct a preliminary assay to determine how this method would check with the photometric procedure and to test the influence of added acetone upon this assay. For each equivalent of sample in our extracts there is five times this amount of acetone. Thus, if a sample is to be tested at a level equivalent to 5 mg., it is necessary to add 0.025 ml. of acetone. For this test, a sample of dried whey containing 23 micrograms of riboflavin per gram was extracted and assayed at two levels. The results are presented in Table I.

This experiment demonstrated that acetone, in the concentrations to be used in our assays, did not influence the growth of *Lactobacillus casei*.

This is in agreement with the report of Snell and Strong (6), who found that alcohol, in amounts up to 20 mg. per tube, did not affect the growth. The calculated riboflavin concentrations were in excellent agreement with our photometric determination.

Numerous chick assays have been conducted for the purpose of developing a satisfactory ration to be used as a basal for testing dairy products (7). Wilgus and coworkers (8) described a chick assay for riboflavin which was later modified by Heiman and Carver (9). Although we were convinced that the ration which we had developed produced chicks which were more nearly normal, an assay was conducted to compare the quantitative determination of riboflavin on our ration and on the ration described by Heiman and Carver (9). The composition of these two rations is as follows:

	Ration I	Washington Ration
Yellow Corn Meal.....	25.0	63.2
Wheat Middlings.....	10.0	20.0
Wheat Bran.....	10.0	5.0
Ground Oats.....	10.0	—
Oatmeal.....	27.5	—
Lactose.....	5.0	—
Casein.....	9.0	8.1
CaCO ₃	1.0	0.2
Bone Meal.....	0.5	2.7
Salt.....	1.0*	0.5
Cod Liver Oil.....	1.0	0.3

* Containing 1.25% MnSO₄ · 2H₂O

A sample of dried whey containing 28 micrograms of riboflavin per gram was selected for assay. Single Comb White Leghorn chicks were fed the respective basal rations for a two-week depletion period and were then evenly distributed into twenty groups of fifteen chicks each. Duplicate pens were used for each supplement and the final weights were averaged separately for the two sexes. The results are presented in Table II.

Good agreement was obtained with these two rations, since in both cases the dried whey was found to contain 32 micrograms of riboflavin per gram. The fact that this value is too high, when compared with the photometric test, can be explained as being due to a lack of more than one factor in both rations. Heiman and Carver found a linear relationship between growth and amount of dried whey fed. Since riboflavin

does not produce this linear response, it can be assumed that whey serves as a source of some of the factors which are missing. We have previously noted that Ration I is deficient in more than one factor and that it can be made more nearly adequate by supplementing with riboflavin-free liver extracts (7). Because of the fact that the chicks on Ration I feathered out more smoothly and showed less tendency toward cannibalism, it was decided to use this ration in the comparative assays.

TABLE II
Basal Rations for Chick Assays

Supplement	Amounts per 100 grams	Sixth-week weight in grams Ration I
None.....	0	133
Riboflavin.....	40 γ	175
“	80 γ	221
“	120 γ	280
Dried Whey.....	2 g.	203
		Washington Ration
None.....	0	168
Riboflavin.....	60 γ	226
“	120 γ	276
“	180 γ	289
Dried Whey.....	3 g.	256

*Experiment I.—Comparative Assay of Four Samples of Buttermilk
from Sweet Cream*

Heiman and Carver found that sweet cream buttermilk contained 60–70% more riboflavin than neutralized dried whey (9). We have also observed a high concentration of riboflavin in this product when tested by our photometric procedure. Hand and Sharp (10) have reported that the distribution of riboflavin between cream and skim milk is dependent upon the previous heat treatment of the milk.

The concentration of riboflavin at the surface of the fat globule may be illustrated by some results obtained upon specially prepared samples of buttermilk (11). These samples originated from two lots of cream containing respectively 25% butterfat and 40% butterfat. The buttermilk prepared from the former sample contained 27 micrograms of riboflavin per gram, while the product obtained from the second sample contained 43 micrograms per gram.

Four samples of buttermilk from sweet cream were selected for assay in this experiment. The extracts used in making the photometric tests were diluted to a convenient volume for the microbiological assay and tested at a level equivalent to 5 mg. of the original powder. Six tubes were used for each sample.

The rat assay was carried out using the following ration:

Casein.....	20%	
Cornstarch.....	68	
Washed Butter.....	8	
Inorganic Salt Mix.....	4	
	—	
	100%	
Vitamin A.....	30	U.S.P. XI Units per gram
Vitamin D.....	8	U.S.P. XI Units per gram
Vitab Supplement 0.5 ml. daily		

TABLE III

Comparative Assay of Four Samples of Buttermilk from Sweet Cream

Method of Assay	Riboflavin in micrograms per gram			
	Sample 1	Sample 2	Sample 3	Sample 4
Photometric.....	30	31	29	30
Microbiological.....	46	50	40	45
Rat.....	58	52	52	49
Chick.....	32	35	39	31
		After 1 year		
Photometric.....	31	30	25	29
Microbiological.....	34	32	28	32

Synthetic riboflavin was fed at a level of 3 micrograms per day as a positive control. The buttermilk powders were furnished at a level of 125 mg. per day.

The chicks were depleted for two weeks on basal Ration I and were then given the same ration supplemented with extracts from dried pork liver at a level equivalent to 2%. The preparation of these extracts has been previously described (7). The positive control received 1 microgram of synthetic riboflavin per gram of ration. The buttermilk powders were fed at a 3% level. The results of this assay are presented in Table III.

In this assay, it was noted that the supplement for the rat assay was not readily consumed. The growth of the rats receiving buttermilk powders so greatly exceeded three grams per week that an accurate determination of riboflavin was impossible. The values, as given in the table, are merely a rough estimate, assuming a linear response to increasing amounts of riboflavin. This, of course, does not actually occur, but

the calculated values have been included to indicate the degree of error in this experiment.

In the chick assay, in spite of the inclusion of liver extracts in the basal ration, two of the samples produced results which were considerably higher than those found by the photometric determination. The chief criticism of this test lies in the rapid growth of the negative controls, with resulting decrease in sensitivity. Part of this difficulty may be due to contamination of the liver extracts with some residual riboflavin. In any case, it was hoped that in future tests the liver extracts might be replaced with crystalline supplements.

The microbiological assay produced extremely discordant results. From time to time, it has been noted that freshly prepared milk powders produce an extra growth stimulation which disappears on standing. As will be noted in Table III, these four samples gave results which were more nearly in agreement with the photometric test after the powders

TABLE IV

Effect of Storage of Milk Powders on the Microbiological Assay for Riboflavin

Method of Assay	Riboflavin in micrograms per gram
Photometric...	37
Microbiological (Extracted in plain glass)....	50
“ (“ “amber “).....	46
“ (“ “painted “).....	48
“ (“ “plain “) after 1 month.....	37

had stood at room temperature for about one year. In another experiment, a sample of buttermilk from sweet cream was assayed in triplicate to determine whether destruction of riboflavin occurred during the extraction procedure. All extractions were made in a dark room which was illuminated by two 40 watt bulbs. One sample was extracted in plain glass, the second in an amber flask and the third in a plain glass flask which had been heavily coated with shellac. After one month, the assay was repeated using a plain glass flask for extraction, since no destruction was observed in the previous test. The results of these tests are presented in Table IV.

Experiment II.—Comparative Assay of Two Samples of Buttermilk from Sweet Cream

After several years' experience with these four methods of assay, various improvements were made in the basal diets. Also, a modified rat

assay had been described which appeared to give excellent checks with the microbiological assay (12). Therefore, we decided to repeat our comparative test using two fresh samples of buttermilk from sweet cream.

The microbiological assay was modified by the inclusion of the following supplements in the basal medium: hydrolyzed casein, lactose, biotin, guanine, and a specially prepared liver extract (11). It has been reported that lecithin is also a growth stimulant for *L. casei* (13). Since buttermilk powder contains considerable lecithin, we conducted assays with and without the addition of lecithin to the basal media. The lecithin was added at a level of 50 micrograms per tube.

The chick ration was altered by this time to include more of Vitamins A, E, and K, more protein and more fat. This was accomplished by including 15% of peanut meal and 2% each of soybean oil and a hexane extract of alfalfa. This is Ration 13 described in a previous paper (7). In place of the liver extracts, we

TABLE V
Comparative Assay of Two Samples of Buttermilk from Sweet Cream

Method of Assay	Riboflavin in micrograms per gram	
	Sample 5	Sample 6
Photometric.....	32	32
Microbiological without lecithin.....	51	51
“ with “	52	50
Chick.....	40	42
Modified Bourquin-Sherman.....	38	<38
Rat Assay (Wisconsin).....	42	33

used a supplement of 1 microgram of thiamin, 3 micrograms of pyridoxin and 7 micrograms of calcium pantothenate per gram of ration.

In the rat assays, we now used sucrose to replace cornstarch and Crisco to replace butterfat. The rice polish concentrate (Labco) was diluted with water and treated with fuller's earth to remove residual riboflavin. After concentrating to a convenient volume, it was fortified with 11 micrograms each of thiamin and pyridoxin per ml. of solution. The second rat assay was conducted according to the published procedure (12). The results of this test are presented in Table V.

Identical curves were obtained in this experiment with and without the addition of lecithin to the basal media. Similarly, identical results were obtained for the two buttermilk powders when assayed with and without the addition of lecithin. Possibly, these results may be explained by the lecithin content of the liver extract which we include in the basal medium. A recent analysis has shown that, in the concentration which we have used, this supplement may furnish as much as 150 micrograms of lecithin per tube. Because of the extremely high results obtained in this assay,

we repeated the microbiological test two weeks later and found 39 and 40 micrograms of riboflavin per gram. These results were more nearly in agreement with the other biological assays, although still much higher than the photometric determination had shown.

The chick assay was conducted with duplicate pens using a 2% level of the unknowns and one microgram of synthetic riboflavin per gram as a positive control. Although the growth in this trial was not equal to that in Experiment I, in which liver extracts were used, it was comparable with that obtained in the preliminary experiment. The calculated riboflavin concentrations would indicate that none of the crystalline supplements tested in this experiment are responsible for the increased growth observed with milk powders.

In the modified Bourquin-Sherman assay, it was found that 1.5 micrograms of riboflavin daily produced growth slightly in excess of 3 grams per week. However, in this test we obtained entirely comparable results from the unknowns, since the dose level had been reduced from 125 mg. to 40 mg. per rat per day. The rats receiving the Wisconsin diet showed symptoms of pantothenic acid deficiency, in spite of the inclusion of a liver extract in the ration. Therefore, a supplement of 100 micrograms of calcium pantothenate was added to the other crystalline vitamins at the end of the depletion period.

Experiment III.—Effect of Fat-Soluble Materials on the Comparative Assay of Buttermilk from Sweet Cream

In a preceding paper, it was shown that chick basal ration 13 is not seriously deficient in inositol, pyridoxin, or pantothenic acid. Preliminary evidence had indicated that the growth-promoting effect of one of the liver extracts was due to choline, but more recent experiments with pure choline have failed to show any marked growth stimulation in chick assays (14). While there have been some indications of other water-soluble growth factors required by the chick, it seemed necessary before proceeding further, to reconsider the adequacy of the proteins in this diet. A supplement of gelatin and cystine was found to improve this ration (14). Consequently, in this experiment, the chick ration was modified to include these two supplements while cystine alone was added to the rat diet.

The previous experiment had demonstrated that lecithin was not the factor which produced extra growth in our microbiological assays. It has also been claimed that other fat-soluble materials may act as growth

stimulants for *L. casei* (13). A preliminary attempt to extract these materials from dried milk products indicated that the process was extremely slow, presumably due to the coating about the fat globules. Andrews (15) has suggested preparing extracts in the usual manner and then treating the extracts with ether to remove fats. This procedure appeared to give excellent results when applied to dried milk products.

To demonstrate the improvement in results which is obtained by this treatment, the two samples from the previous experiment were assayed twice, using the original extracts and the fat-free extracts in each assay. These results are presented in Table VI. Since these two samples had been found to contain 32 micrograms of riboflavin by our photometric method, it is apparent that ether extraction brings these two methods of assay into essential agreement.

It did not appear probable that fats could be the cause of the high results obtained in the animal assays, because of the low concentrations

TABLE VI
Removal of Bacterial Growth Stimulants by Ether Extraction

Trial	Method	Riboflavin in micrograms per gram	
		Sample 5	Sample 6
1	Regular extraction	42	41
1	Fat-free extract	32	31
2	Regular extraction	39	39
2	Fat-free extract	30	28

which are fed in assaying buttermilk powders. However, a series of 20 g. samples of buttermilk from sweet cream were extracted until a total of four pounds had been treated. The combined extracts were treated with three portions of ether, and the water-soluble and ether-soluble materials were assayed in comparison with the original buttermilk powder.

The chicks for this assay were depleted as usual for a two-week period. During the four-week assay period, the basal ration was modified by the substitution of 4% gelatin and 0.2% cystine for an equal amount of casein. The rats also received a supplement of 0.2% cystine. The results of this assay are presented in Table VII. The amount of material tested in the various methods of assay presented in Table VII are expressed as follows:

1. Photometric and microbiological procedure—15 gram sample extracted.
2. Chick assay—% sample in ration.
3. Rat assay—mg. sample per rat per day.

The extracts were fed at a slightly higher level in this experiment, because it was thought at first that some loss had occurred during the manipulation of this

large batch. However, later comparisons of photometric and microbiological results failed to demonstrate any significant loss.

This sample of buttermilk was tested when first received, and reasonably good checks were obtained between the photometric and microbiological assays without making use of the ether extraction procedure. Perhaps this may account for the excellent results obtained in the rat assay. However, the chick assay on this sample showed the greatest error that we have observed.

Part of this error is undoubtedly due to using a 3% instead of a 2% level for assay. From microbiological assays of the basal ration and of the buttermilk powder, it was calculated that a 3% level of the latter would result in a total of 2 micrograms of riboflavin per gram of feed. This is supposed to be suboptimal (16) and represented an intermediate

TABLE VII

Comparative Assay of Fractions Obtained from Buttermilk from Sweet Cream

Method of Assay	Fraction tested	Amount	Riboflavin in micro- grams per gram
Photometric.....	Extract	15 g.	33
Microbiological.....	"	15 g.	35
"	Fat-free extract	15 g.	29
Chick.....	Powder	3%	>100
"	Fat-free extract	3.7%	84
"	Combined "	3.7%	47
Rat.....	Powder	45 mg.	33
"	Fat-free extract	55 mg.	33
"	Combined "	55 mg.	33

value for this assay, since synthetic riboflavin was fed at levels of 0.75, 1.5, and 3.0 micrograms per gram. Numerous tests have shown that almost quantitative extraction of riboflavin is obtained by the procedure employed in this experiment. The difference observed in this assay between the original powder and the extract is of doubtful significance. In any case, the growth obtained with the extract demonstrates that the factor which is complicating this assay is also removed by acid-acetone extraction. The ether extract appeared toxic, since less growth occurred when this material was fed in combination with the fat-free extract.

The results of this assay would appear to be inconclusive so far as the Bourquin-Sherman technique is concerned. The chick assay again points to unidentified factors in dried milk products as we have previously suggested (17).

SUMMARY

Numerous experiments have demonstrated that buttermilk from sweet cream contains factors which produce erroneous results when assayed for riboflavin by biological methods. Good agreement can be obtained between the photometric test and the microbiological assay if fat-soluble factors are removed before applying the latter procedure, and the basal medium is supplemented as described. The rat assay may also be improved by the ether extraction, but sufficient data to prove this point is lacking. Further studies will be required to explain the very marked growth stimulation observed in chick assays.

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The Effect of Dry Grinding on the Properties of Proteins*

2. Studies on Casein

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INTRODUCTION

In a previous communication (1) the effect of dry grinding on native ovalbumin, denatured ovalbumin, and coagulated ovalbumin was reported. Soluble proteins are converted into a less soluble form, and insoluble proteins are converted into water-soluble fragments. It is evident that dry grinding produces definite changes in the protein molecule. The effect of dry grinding on casein is presented.

EXPERIMENTAL

25 grams of casein (Code 1559, General Chemical Co., N. Y.) the purity of which was undetermined, were washed completely free of water-soluble protein and placed in a ball mill of 8 liters capacity and ground for 48 hour periods. Four water-soluble fractions were obtained. Upon these four water-soluble proteins and the water-insoluble residue remaining after four successive periods of grinding, the action of rennin and the phosphorus, nitrogen and tryptophan content were determined. The action of rennin was carried out on the fractions dissolved in a buffer of pH 6.5. Phosphorus was determined by the Neumann-Pemberton (2) method and nitrogen by the Kjeldahl method. The May and Rose (3) method was used to determine tryptophan (Table I).

RESULTS

The water-soluble protein obtained by the action of grinding during the first 48 hour period had a higher phosphorus content and a lower

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percentage of tryptophan than that of unground casein; rennin acted on this fraction yielding a loose clot. The other fractions were not affected by rennin but contained more phosphorus than native casein. All the water-soluble fractions were low in tryptophan. The low tryptophan content of these fractions may be taken to indicate that the grinding process breaks specific linkages in the casein molecule, splitting off water-soluble fractions having a low concentration of this amino acid or that tryptophan is destroyed by the grinding process. There is little difference in the percentage of nitrogen in the different fractions.

The first water-soluble fraction is precipitated by acetic acid. On cooling the hot solution, the protein reprecipitates, a maximum amount

TABLE I

Chemical Differences in the Water-Soluble Fractions, Native Casein, and Residue

Sample	Rennin Action	Phos- phorus <i>per cent</i>	Nitrogen <i>per cent</i>	Trypto- phan <i>per cent</i>	Water-soluble Protein from 25 g. Casein
					<i>g.</i>
Washed Casein	+	0.85	15.70	2.2	25
1st	+	1.15	15.28	0.44	5.5
2nd	—	1.19	15.61	0.47	3
3rd	—	1.26	15.61	0.64	2
4th	—	1.05	15.04	0.69	2.5
H ₂ O Insol. residue after 4 grindings ..	+	0.67	15.35	1.38	16.1

forming at 50–60°C. When the solution has reached room temperature most of the protein has redissolved.

The second water-soluble fraction is not precipitated by acetic acid but in water solution gives the same type of reaction as described for the first fraction. In acid solutions (pH 4.5) a higher temperature is required to bring about coagulation than that necessary when the protein is dissolved in distilled water. There is also a lesser quantity of coagulum formed in solutions of this acidity.

The third and fourth water-soluble fractions are not precipitated by acid and do not coagulate in solutions of acidity below pH 4. These fractions dissolved in distilled water are coagulable.

The fractions all contain dialyzable proteins. The water-soluble proteins are precipitated from solutions in water by trichloroacetic, picric acid, mercuric chloride, phosphotungstic acid, and half saturated am-

monium sulfate. Although the first fraction is precipitated by acetic acid, the other fractions are not precipitated by acids or alkali. All water-soluble proteins give a pink-violet biuret test, indicating a smaller molecular size.

The water-soluble proteins are not precipitinogenic but produce anaphylactic sensitization in guinea pigs.

Comparison of Tryptic Digestion Rates of the Casein Fractions

Solutions of the proteins were made up in a phosphate buffer at pH 8.5. The concentration of protein in all of the digests was the same and equal to 31.6 mg./cc. The enzyme used was trypsin (Fairchild) and the

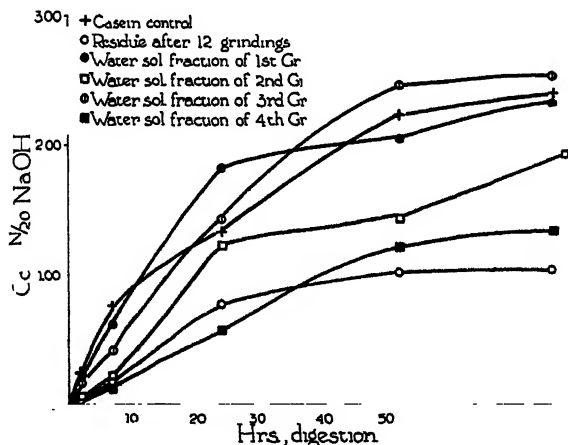


FIG. 1

Comparison of Rates of Digestion of Water-Soluble Proteins of Casein and Native Casein by Trypsin

amount added was such as to give a concentration of 3.16 mg./cc. 3 cc. of 5 per cent thymol in alcohol for 50 cc. of digest was used as a preservative. Digestion was carried out at 37° C. The rate of hydrolysis was followed by the Sørensen titration using N/20 NaOH (Fig. 1).

The results indicate that prolonged grinding with removal of the water-soluble protein formed leaves a residue which is only slowly digested by trypsin. The water-soluble proteins are all digested much more readily than the water-insoluble residue, although the water-soluble protein of the fourth grinding is only slowly attacked by the enzyme during the first 34 hours of hydrolysis. The water-soluble fraction of the first grinding is

hydrolyzed at a faster rate during the first 45 hours than is native casein. The hydrolysis of this fraction has almost reached its maximum at the end of this period. In the case of the native protein maximum hydrolysis requires at least 50 hours.

It is quite evident from these studies that the water-soluble proteins produced by grinding are different from one another, although their content in nitrogen, phosphorus, and tryptophan, and the effect of heat on their solutions indicate some degree of similarity.

Water-Soluble Casein in Nutrition

500 grams of dry Harris vitamin free casein, extracted free of water-soluble protein, were ground in a ball mill of 8 liters capacity for successive periods of 48 hours each. After each 48 hours the ground powder was removed from the mill and extracted with distilled water until free of the water-soluble protein produced by the grinding. The dried insoluble

TABLE II
Analysis of Proteins Used in Nutritional Studies

Fraction	Phosphorus <i>per cent</i>	Sulfur <i>per cent</i>	Tryptophan <i>per cent</i>
Water-Soluble	1.27	0.45	0.53
Water-Insoluble	0.68	0.089	1.86
Original Casein	0.85	0.78	2.20

residue was again subjected to further grinding. By 12 successive grindings 40 grams of water-soluble protein were obtained. This was utilized for the study of the nutritional value of the water-soluble component as compared with that of unground casein and the water-insoluble residue remaining after the grinding process. The phosphorus, sulfur, and tryptophan content of these fractions have been determined (Table II).

It was desirable to determine the adequacy for growth of the water-soluble protein produced from casein since it gives some indication as to its content in the essential amino acids.

White mice were used in these experiments because of the limited quantity of the water-soluble casein available for use in making up the diet. The Sherman-Sandels diet was used and contained casein 18 g., cornstarch 52 g., salt mixture 4 g., butterfat 7 g., autoclaved yeast 15 g., and cod liver oil, 3 g.

Three mice were fed on a diet with the combined water-soluble protein obtained by successive grinding extraction and grinding of Harris casein.

Another three mice were given a diet with residue remaining after 12 successive grindings as the protein in the diet. A third series of three mice were given a diet with unground casein which had been completely extracted free of water-soluble proteins. The growth curves for the diets are given in Fig. 2. The phosphorus, sulfur, and tryptophan of the water-soluble protein and the water-insoluble residue after 12 grindings were determined by methods described previously (Table II).

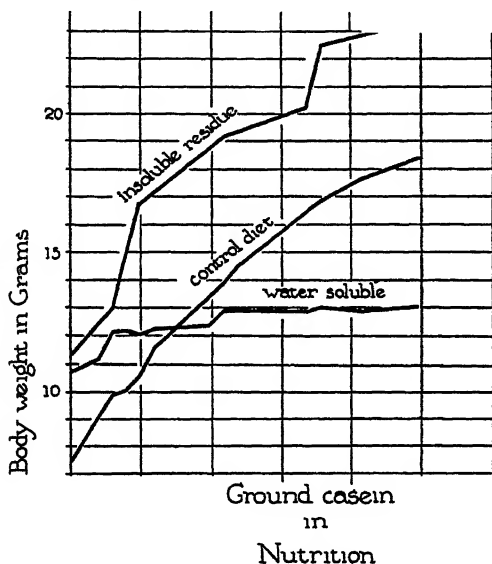


FIG. 2

The Value of Water-Soluble Casein in Nutrition of Mice Compared With That of the Insoluble Residue and Native Casein

The results definitely show that the water-soluble casein is nutritionally deficient since it does not support growth in mice. The insoluble residue was just as effective in maintaining the animal in good health and for growth as the native casein. It would thus appear that prolonged grinding does not affect the essential amino acids greatly since a complete lack of any of these in the ground residue would have produced a deficient diet.

Totter and Berg (4) have shown that mice will grow on a diet previously deficient in tryptophan if the natural amino acid is added to the diet in a concentration of 0.1% . The addition of 0.2% of the *l*-amino acid tryptophan to the deficient diet produced much better growth. Fig. 2 indicates that the water-soluble protein of ground casein, although capable of maintaining the animal in good health and at a relatively constant weight, was at least partially deficient from a nutritional standpoint. Since the tryptophan content of the diet containing the water-soluble protein was approximately 0.1% , the lack of growth was due to a partial deficiency of some other amino acid or acids, other than tryptophan in the diet. Because of the small number of animals used in this experiment, further studies will have to be carried out before definite conclusions can be made.

How Does Grinding Produce Degradation of the Protein Molecule?

It has been shown that water-soluble proteins are converted by dry grinding into an insoluble form. The dry grinding of proteins insoluble in water produces water-soluble fragments. From the available data the conclusion seems justifiable that definite linkages in the protein molecule are attacked.

The water-soluble proteins, if we assume a Wrinch structure, would become insoluble through an unfolding of the cage molecule, similar to that which occurs as a result of surface spreading.

The transformation of the soluble protein into the insoluble form is apparently only a preliminary step in the alteration of the protein molecule by the action of dry grinding. The linkages broken at this point must be the weak secondary valence bonds, thus producing intramolecular changes. The grinding of these insoluble proteins and those insoluble in the native state produce further changes which lead to the splitting off of water-soluble fragments. The linkages involved here are not known. In studies on gelatin (see page 353), it will be shown that detectable peptide splitting did not occur. The possibility exists that while wholesale peptide splitting did not take place, such bonds could have been broken but in numbers too few to be determined.

That the soluble protein is produced by the action of microorganisms acting during extraction of the dry ground powder is unlikely since these soluble fractions are immediately extractable by distilled water.

It would be interesting to know by what mechanism this fragmentation of the protein molecule is brought about. Routh (5) has discussed

the rôle played by the composition of the mill and balls on the grinding process. The temperature does not increase since the mill and its contents are only slightly warmer than the external atmosphere. However, at the point of contact of the balls a large amount of energy as heat may be liberated and this may be one of the factors influencing the changes produced. The odor which is observed with all proteins after they have been ground may be associated with this heat effect. The balls as they strike each other may constitute a shearing force which 'slices' the protein into at least two fragments, one of which is at least soluble in water.

CONCLUSIONS

1. Water-soluble proteins are produced when casein is dry ground.
2. The water-soluble proteins contain a higher content of phosphorus than native casein.
3. The fractions all have a low tryptophan content.
4. The first water-soluble protein fraction was acted upon by rennin but the others were not.
5. The water-soluble protein is partially deficient but maintains mice in constant weight.

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The Effect of Dry Grinding on the Properties of Proteins*

3. Gelatin

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INTRODUCTION

Alsberg and Griffith (1) state that dry grinding renders gelatin cold-water-soluble. Solutions of ground gelatin in distilled water form gels after standing for some time. Alsberg considers the changes observed to be of a purely physical nature. Boissevain (2) repeated these studies and found that gelatin ground in a ball mill for 96 hours became perfectly water-soluble. A 10 per cent solution of this gelatin would not gel even after standing in the ice box for a week. Boissevain explains the changes by assuming that hydrolysis of the gelatin into peptone had occurred. Short periods of grinding apparently had no effect on the gelatin properties of this protein.

EXPERIMENTAL

Fifty grams of Knox granular gelatin were placed in a ball mill of 7 liters capacity and the mill rotated at about 100 R.P.M. Samples were removed at intervals of time, the total time of grinding being 72 hours.

Solutions of definite concentrations of the ground samples were prepared by dissolving the necessary quantity of the dried protein in distilled water at room temperature. Unground gelatin was dissolved at 60°C. and allowed to cool to room temperature. The solutions were placed in a water bath at 5°C. and the time required for gel formation was determined. The results are tabulated in Table I.

To determine whether the change in solubility was due to a hydrolytic

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process, as suggested by Boissevain, the Sørensen titration was carried out upon 1 gram samples of protein dissolved in distilled water using 0.05 *N* NaOH (Table II).

It is generally known that gelatin heated about 70°C. for a number of hours loses its ability to gel. Unground gelatin which in 2 per cent concentration formed a gel in 3 minutes required 31 minutes to gel after heating for 18 hours at 80°C. Gelatin ground for 7 hours and largely

TABLE I
Effect of Grinding Gelatin on Time for Gel Formation

Hours Grinding	Time for Gel Formation Concentration of Protein			
	2%	3%	4%	5%
0	3 min.			
7	3 min.			
22	12 min.			
30	14 min.			
48	24 hrs.	6 hrs.	1 hr.	17 min.
54	"	"	"	15 hrs.
72	"	*	"	"

* No gel formed even after 10 days at 5°C.

TABLE II
Formal Titrations of Ground Gelatin

Hours Grinding	cc. 0.05 <i>N</i> NaOH per g.
0	5.04
7	5.05
22	5.07
30	4.98
48	5.00
54	5.03
72	4.95

soluble in cold water and which also formed a gel in 3 minutes required standing for 2 hours before a gel formed after being similarly treated.

RESULTS

It is evident that the longer the period of grinding the more drastic is the effect on the protein molecule. The grinding of gelatin for 7 hours has no effect on its ability to form a gel but there is a marked increase in solubility in water at room temperature. That there are other differences is indicated from the results obtained by heating the ground

and unground samples of gelatin, the former requiring 4 times as long for gel formation as the latter.

The formal titration values indicate that there was no appreciable splitting of peptide bonds. Boissevain explained his results by assuming a hydrolytic process with conversion of the gelatin into peptone. The change in the physical properties of the gelatin could be explained by the assumption that peptide bonds may have been broken. The splitting of secondary valence bonds such as hydrogen bonds and disulfide bonds of cystine might produce similar results.

DISCUSSION

The ability of gelatin solutions to form gels is according to Katz and Gerngross (3) dependent on the arrangement of the molecules into a definite structure. Since short periods of grinding have little effect on gelatin, other than to increase its solubility in cold water, it would seem that the early changes produced are not drastic but merely serve to open up linkages leading to a greater number of polar groups in the molecule and thus increased solubility. Prolonged grinding produces more deep seated changes.

The increased solubility cannot be explained on the basis of a mechanical process bringing about a greater availability of the material to solvent action. Nor is it possible to consider the differences in ground and unground gelatin as due to hydrolytic action alone as Boissevain believes.

SUMMARY

1. The effect of grinding gelatin is to convert it into a cold water-soluble protein.
2. The gel formation time increases with increase in the time of grinding.
3. There is no increase in the formal titration value during grinding.

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The Effect of Dry Grinding on the Properties of Proteins*

4. Human, Beef, and Hog Coagulated Hemoglobins

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INTRODUCTION

In previous papers (1) we have described the effect of dry grinding on various forms of ovalbumin, gelatin, and casein. In this paper we report the effect of dry grinding on human, beef, and hog coagulated hemoglobins.

EXPERIMENTAL

The method of Marshall and Welker (2) was used for the preparation of the hemoglobin. The clear dilute (0.2 per cent) solutions of hemoglobin were adjusted to pH 6.8 with molar K_2HPO_4 and the solutions placed in a boiling water bath for 30 minutes. The dark brown coagula were filtered off, washed with boiling water, and spread upon glass plates to dry.

The dry proteins were separately ground in a ball mill of 8 liters capacity for successive periods of 48 hours each. After each 48 hour period, the water-soluble protein was extracted from the powder with distilled water. The water-soluble protein was obtained in dry form by passing dry warm air at 38°C. over the solutions. The insoluble residues were similarly dried before being subjected to further grinding.

From coagulated human hemoglobin we prepared 8 water-soluble fractions, 4 from coagulated beef hemoglobin and 2 from coagulated hog hemoglobin.

The percentages of nitrogen, iron, tyrosine, and tryptophan of these fractions were determined. Nitrogen was determined by the Kjeldahl

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method, iron by MacFarlane's method (3) (the Pulfrich Photometer was used instead of the ordinary colorimeter), tyrosine and tryptophan by the Folin and Marenzi method (4). The amount of dialyzable nitrogen was determined by exhaustively dialyzing solutions of known concentration through Visking dialysis shells (Table I).

TABLE I
Water-Soluble Fractions of Coagulated Ground Hemoglobin

Sample	N ₂	Fe	Tyrosine	Trypto- phan	Amount of Water-Sol. Extract by H ₂ O <i>per cent of orig.</i>	Diffusible Nitrogen <i>per cent</i>
<i>Human Hemogl.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
1st	15.54	0.2505	2.47	0.41	16.6	77.95
2nd	15.30	0.2709	2.12	0.72	25	95.09
3rd	15.35	0.276	1.98	0.76	8.7	84.53
4th	15.21	0.420	2.09	0	6	93.36
5th	14.90	0.420	2.02	0	5	88.12
6th	14.52	0.370	1.86	0	8.3	69.93
7th	12.79	0.390	1.62	0	5.4	83.79
8th	11.72	0.362	1.61	0	5	78.25
<i>Ox Hemogl.</i>						
1st..	15.11	0.174	2.74	0.61	21.5	
2nd	15.20	0.370	2.42	0.72	32.5	
3rd.	14.65	0.354	2.45	0	10	
4th...	13.25	0.285	2.75	0.75	10	
<i>Hog Hemogl.</i>						
1st..	15.55	0.228	1.85	0.72	17.5	
2nd....	15.31	0.336	2.42	0.54	15	

RESULTS

As seen in Table I the water-soluble fractions contain iron in variable amounts. They all give the benzidine reaction. The fact that the hematin is soluble in water indicates that the prosthetic group is not split from the protein moiety. The manner in which the hematin is attached to the protein remains unknown but it is quite definite that this linkage is not split by the grinding procedure.

The water-soluble proteins contain dialyzable protein, are non-coagulable by heat and require concentrations of 50% or more of alcohol for precipitation. They are precipitated by mercuric chloride, picric

acid, trichloroacetic acid, and by half saturation with ammonium sulfate. They are soluble in acids and alkali. The indications are that the fragments produced by grinding are small in molecule size, probably that of primary and secondary proteoses. The water-soluble fractions do not give rise to precipitin antibodies and do not react with native hemoglobin antisera.

The nitrogen content decreases with successive fractions which may be interpreted as a loss of ammonia by deamination during the grinding process. This seems also to be true for the tyrosine in human hemoglobin while that of the soluble fractions of beef hemoglobin is fairly constant.

The distribution of tryptophan in the fractions is also quite interesting. This amino acid is absent in the last four fractions of human hemoglobin and in one of the water-soluble fractions of the beef hemoglobin.

79% of the coagulated human hemoglobin was converted into water-soluble protein in 384 hours grinding. If the grinding had been continued all of the coagulated hemoglobin might possibly have been obtained as soluble protein. 75% of the coagulated beef hemoglobin was converted in 192 hours grinding, while 32.5% of the coagulated hog hemoglobin became soluble in 96 hours.

The water-soluble fractions from human hemoglobin contain at least 70% dialyzable nitrogen, thus indicating that they are small molecular fragments. The hematin remained unaffected by 300 hours of continuous grinding since it was possible to recover from the ground powder the theoretical yield of hematin by recrystallization from a chloroform and quinine solution.

Spectrographic Analysis

The analysis was carried out using a Hilger Quartz Spectrograph, spectra being photographed upon Eastman Orthochromatic plates. Ultra violet spectra were obtained by the use of a Kromayer mercury arc lamp. All plates contain one comparison spectrum transmitted through an empty cell and a series of spectra through the same cell filled with the material being studied. The time of exposure was 50 seconds, the concentration of protein was 0.05 per cent. The light portion of the plates are areas affected by transmitted light. A normal spectrum of the native hemoglobin and of hematin (.002 per cent) dissolved with the aid of alkali is included for comparison. A portion of the visible spectrum is faintly seen at about 540 and 570 $\mu\mu$ (Figs. 1, 2, 3).

RESULTS

The water-soluble fractions seem to differ from native hemoglobin mainly in the region of the ultraviolet from 313 $\mu\mu$ to 264 $\mu\mu$. At 313 $\mu\mu$ they absorb more ultraviolet light than does the native protein. The water-soluble fractions of coagulated human hemoglobin absorb more strongly at 297 $\mu\mu$ as we go from the 1st to the 8th grinding. This is also true at 280, 276, and 264 $\mu\mu$. At 258 $\mu\mu$ and 248 $\mu\mu$ absorption by the ground fraction is complete in all cases except that of the first fraction, less than 1% being transmitted at 248 $\mu\mu$.

The similarity in the ultraviolet absorption of all three hemoglobin fractions, suggests the possibility of the existence of similarity in the structure of the molecules of hemoglobins studied. The protein fragments split off by the grinding process may possibly have a similar structural arrangement.

DISCUSSION

It is apparent that the grinding process brings about changes in the three coagulated hemoglobins similar to those observed in our study of coagulated ovalbumin. As in the case of the latter protein a stepwise degradation of the protein molecule is effected. The high quantity of diffusible nitrogen and the precipitability of the protein in the water-soluble fractions by various reagents indicates that small molecular weight particles are present. Data from the Laboratory of Dr. Hans Neurath, received after the paper on ovalbumin was reported, on diffusion measurements of the water-soluble fractions of coagulated ovalbumin indicates that the particles of lowest molecular weight are 10,000-12,000.

CONCLUSIONS

1. Water-soluble protein is produced when coagulated hemoglobin is dry ground.
2. The nitrogen and tyrosine content of the water-soluble proteins decreases with successive grinding.
3. There occurs during grinding a most decided degradation of the protein molecule.

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The Production of a Creatinine Oxidase and a Creatine Anhydrase from Rat Feces

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INTRODUCTION

One of the most significant advances in the study of creatine and creatinine metabolism in recent years was the isolation of specific creatinine decomposing enzymes from soil bacteria by Dubos and Miller (1, 2). By the use of these enzyme suspensions one can be certain that the substance responsible for the development of the Jaffe reaction with alkaline picrate is either creatine or creatinine. A review of these adaptive enzymes has been published by Dubos (3). One of their chief characteristics is that they are remarkably specific toward the substrates which have stimulated their production.

A quantitative recovery of creatine and creatinine is never obtained when these substances are ingested. It is reasonable, therefore, to believe that they may, in part, be decomposed by intestinal bacteria. A study was made using a suspension of rat feces from which a creatinine oxidase under aerobic conditions and a creatine anhydrase under anaerobic conditions was obtained.

EXPERIMENTAL

Preparation of FE suspensions. 100 g. of fresh feces from rats fed on Purina Dog Chow and water were broken up with a stirring rod in 400 cc. of water in a 600 cc. beaker and incubated for 2 hours at 37°C. Solid material was centrifuged off twice. The supernatant liquid was then distributed into 1-liter Blake bottles, each containing 50 cc. of the following medium (2): 2 g. Bactotryptone, 200 mg. Bacto-yeast, 5 g. creatinine, 5 g. NaCl plus "artificial tap water" (4) to 1000 cc. After incubation of the solutions at 37°C. for 4 days a gray film formed on the sides and bottom of the bottles. On turning the bottles over a clear separation of the film and fluid was obtained. The latter was decanted and the film washed three times with distilled water in the centrifuge. The washed film material was then

evenly distributed into the 15 Blake bottles, and 50 cc. of the following medium was added to each: 5 g. creatinine, 5 g. NaCl, 25 cc. phosphate buffer pH 7 (50 cc. of 0.2 M KH_2PO_4 + 29.63 cc. of 0.2 N NaOH diluted to 200 cc.) + artificial tap water to 1000 cc. After incubation at 37°C. for 4 days the supernatant fluid gave no Jaffe reaction with alkaline picrate (FE_1). Successive transfers of small amounts of the centrifuged film material were then made as above and named FE_2 to FE_3 , etc., and incubated with fresh solutions of creatinine in artificial tap water. At the end of the incubation periods the Jaffe reaction with alkaline picrate was negative in all cases. No attempt was made to identify or isolate the organisms of the film.

RESULTS AND DISCUSSIONS

Rate of Disappearance of Creatinine. It is difficult to compare the rate of decomposition of creatinine with different enzyme preparations.

TABLE I

Rate of Disappearance of Creatinine With and Without FE_7 and NC_4 Enzymes
(1 mg. Creatinine + 5 cc. Phosphate Buffer pH 7)

Incubation at 37°C.	Control Creatinine (after incubation)	Creatinine + 5 cc. FE_7		Creatinine + 5 cc. NC_4	
		Creatinine	Creatinine Disappeared	Creatinine	Creatinine Disappeared
hours	mg.	mg.	per cent	mg.	per cent
$\frac{1}{4}$	0.90	0.58	35.5	0.68	24.4
1	0.90	0.55	38.9	0.66	25.6
$1\frac{1}{2}$	0.93	0.50	46.2	0.62	33.3
2	0.96	0.47	51.0	0.48	50.0
3	0.95	0.35	63.1	0.35	63.1

Some autolysis of the cells occurs on desiccation or when they are allowed to stand under water in the ice chest with subsequent loss of activity. In spite of this difficulty, however, these comparisons were made on two preparations, our FE_7 and desiccated NC (D and M) suspensions, and these results were then compared with those of the original NC suspension of Dubos and Miller (2).

1 mg. of creatinine in 5 cc. of the phosphate buffer at pH 7 was incubated with 5 cc. of FE_7 suspension. At the end of the incubation periods a small amount of creatinine had disappeared in the control flasks. This could be due to oxidation of creatinine or to its transformation into creatine (5). For this reason we calculate the amount of creatinine which disappeared after enzyme action with that present at the end of the test period in each control flask rather than with the amount of creatinine present at the beginning of the experiment (Table I).

On the average 0.26 mg. of creatinine disappeared per hour with FE_7 as compared to 0.20 mg. per hour with our NC_4 suspension. The amount of creatinine disappearing with both cultures was identical at the end of 2 and 3 hours incubation. The increase in the percentage decomposition of creatinine ranged from 36 to 63 for FE_7 as compared to 24 to 63 for NC_4 . These results were then compared with those published by Dubos and Miller (2) for their original NC suspension (Table II).

In the first hour all rates were about the same, but at the end of the second hour the original Dubos and Miller enzyme (NC) destroyed 0.79 mg. of creatinine as compared to 0.43 and 0.48 mg. respectively, with the FE_7 and NC_4 suspensions. Our preparations are, therefore, slightly less active than the original NC suspension of Dubos and Miller.

TABLE II
Comparison of the Decomposition of Creatinine

Enzyme	Hours incubated with enzyme at 37°C.			
	1	1	2	3
	Creatinine Destroyed			
	mg.	mg	mg.	mg.
NC (Dubos and Miller (2))	0 35	0.38	0.79	
FE_7 (Beard)	0 32	0 35	0.43	0.60
NC_4 (D and M) Beard	0 22	0.34	0.48	0.60

Specificity of the FE Enzymes. Our specificity studies are based upon the appearance or disappearance of the Jaffe reaction with alkaline picrate. This technique for our purposes, however, has its limitations since 1 mg. of glycocyamine, after autoclaving for 30 minutes with HCl at 15 lbs. pressure, as well as 1 mg. of methyl hydantoin or hydantoin, do not give the Jaffe reaction with alkaline picrate. These substances could not, therefore, be tested in this connection. Methyl and dimethyl derivatives of creatinine were not tested since they would not occur in body tissues or fluids.

Our FE_7 suspension did not destroy creatine or glycocyamidine. This is interesting in view of the fact that it required only 5 hours for the complete decomposition of glycocyamidine by the original NC suspension of Dubos and Miller (2). It would, therefore, seem that our FE enzyme is even more specific for creatinine than their NC suspension since our enzyme does not grow on or destroy glycocyamidine at all.

EFFECT OF pH ON THE ACTIVITY OF THE FE ENZYME

5 cc. of FE_7 + 1 mg. of creatinine in 5 cc. of the phosphate buffer were incubated at 37°C. for 4 hours at pH ranges from 2.4 to 9.8. The results show that the enzyme acts best at a pH of 7 (Table I), next in alkaline and least of all in an acid medium (Table III).

Growth of FE_7 on Various Substances With Disappearance of Creatinine

The results listed in Table IV show that some growth of the organisms was obtained on creatine and glycine, slightly more on arginine, and most of all on alanine. No growth occurred on the other substances

TABLE III
Effect of pH on Disappearance of Creatinine by FE_7

FE_7	Creatinine	Incubation	Creatinine		pH
			After incubation	Disappeared	
cc.	mg.	hours	mg.	mg.	
5	1.0	4½	0.93	0.02	2.4
5	1.0	4½	0.88	0.07	3.6
5	1.0	4½	0.68	0.27	5.8
5	1.0	4½	0.55	0.40	8.2
5	1.0	4½	0.53	0.42	9.1
5	1.0	4½	0.72	0.23	9.8
0	1.0	4½	0.95		7.0

tested. The organisms grown on creatine destroyed creatinine. None of the other cultures, however, did so. It would seem, therefore, that our enzyme shows a high degree of specificity for creatinine.

Transformation of Creatine into Creatinine by FE_7 under Anaerobic Conditions

Five 200 cc. acid washing bottles were set up. In the first was placed a strong solution of alkaline pyrogallol and was connected to the air jet. The second and third bottles contained distilled water. In the fourth was placed the control creatine solution (10 mg. per 30 cc.) and in the fifth was placed the same amount of creatine together with 5 cc. of FE_7 in 30 cc. After connecting the bottles in a train air was bubbled through the alkaline pyrogallol into all flasks for 30 minutes. Nos. 4 and 5 were then clamped off and placed in the incubator at 37°C. overnight. Next morning an intense Jaffe reaction with alkaline picrate occurred in bottle 5 while only a slight Jaffe occurred in the creatine control bottle 4.

1 mg. of creatine in 10 cc. of artificial tap water was placed in bottles 2 and 3,

and this same amount of creatine with 2 cc. of FE_7 was placed in 4 and 5. Air was bubbled from the jet through alkaline pyrogallol in bottle 1 into these 4 bottles for 30 minutes. At this time all bottles were clamped off and incubated

TABLE IV

Growth of FE_7 on Various Substances With Disappearance of Creatinine¹

Flask No.	Ingredient	FE_7	Growth of FE_7	FE_7 + 1 mg. Creatinine ²
				Creatinine
		cc.		mg.
1	Creatine	2	++	0
2	Glycocyamine	2	0	0.89 ³
3	Methyl hydantoin	2	0	0.93 ³
4	Hydantoin	2	0	0.92 ³
5	Glycocyamidine	2	0	0.90 ³
6	Guanidine acetate	2	0	0.92 ³
7	Glycine	2	++	0.93 ³
8	Urea	2	0	0.92 ³
9	Alanine	2	++++	0.91 ³
10	Arginine	2	+++	0.90 ³

¹ 100 mg. of each substance were dissolved in 50 cc. of "artificial tap water" (2) containing 2 cc. FE_7 suspension and incubated for 4 days at 37°C.

² The whole FE_7 suspension after incubation and centrifugation was incubated for 6.5 hours with 1 mg. creatinine at pH 7 in artificial tap water.

³ 0.1 mg. creatinine disappeared spontaneously, probably due to oxidation (cf. Table 1, Column 2).

TABLE V

Showing the Presence of Creatinine Oxidase and Creatine Anhydrase in FE_7

Bottle	Creatine	FE_7	Creatine "as creatinine"	Creatinine
no.	mg.	cc.	mg.	mg.
2	1.0	0	0.97	
3	1.0	0	0.95	
4	1.0	2		0.0
5	1.0	2		0.0

at 37°C. for 2 hours. The addition of HCl and autoclaving the contents of 2 and 3 gave a quantitative transformation of creatine into creatinine (Table V) showing that, under these conditions, the creatine was not transformed into creatinine during the 2 hour incubation period in the absence of the FE enzyme. At the end of the 2 hour incubation period under anaerobic conditions the contents of 4 and 5

were transferred into open Blake bottles and allowed to incubate for another 2 hours at 37°C. A negative Jaffe reaction with alkaline picrate occurred in these two flasks.

These results prove the presence of a creatinine oxidase under aerobic conditions and a creatine anhydrase under anaerobic conditions in our FE₇ suspension. This behavior is similar to that shown by the NC suspensions of Dubos and Miller (2, 6). A creatinine oxidase, a creatine oxidase, and a creatinine hydrase, have also been shown by the writer to occur in rat tissues, especially in intestine, lung, heart, and kidney (7). With one exception (that of a change of creatine into creatinine in water solution) we have never been able to obtain the transformation of creatine into creatinine *in vivo* or *in vitro* (5). The existence of a creatine anhydrase was never demonstrated although it has been believed for many years that creatine is normally dehydrated into creatinine in the body. It was quite interesting to us to be able for the first time to observe this unusual change and to demonstrate the presence of the enzyme causing it in a suspension made from rat feces.

SUMMARY

1. Using the technique of Dubos and Miller a creatinine oxidase, which destroys creatinine under aerobic conditions, and a creatine anhydrase, which transforms creatine into creatinine under anaerobic conditions, have been isolated from a suspension obtained from fresh rat feces.

2. The creatinine oxidase acts best at a pH of 7, next in alkaline and least of all in an acid pH.

3. At pH 7, when incubated at 37°C. with creatinine, it destroys 0.26 mg. of creatinine per hour. This rate compares favorably with our desiccated NC suspension, and with the original NC suspension of Dubos and Miller.

4. Using the Jaffe reaction with alkaline picrate the creatinine oxidase shows a high degree of specificity for creatinine, since it does not destroy creatine or glycocyamine under aerobic conditions.

5. The organisms which grow on creatine will also decompose creatinine. Some growth of these organisms occurred on glycine and creatine, more on arginine, and most of all on alanine, each as the sole source of nitrogen and carbon. Growth did not occur on glycocyamine, glycocyamine, hydantoin, methyl hydantoin, guanidine acetate, or urea. Only those suspensions grown on creatine or creatinine destroyed the latter.

ADDENDUM

Since this manuscript was submitted for publication a *creatinine oxidase* has also been produced from the mould that grows on top of rat urine when kept in a beaker at room temperature for 3 days. The suspension does not, however, transform creatine into creatinine. The technique used was the same as described in the above paper. Tests for the specificity of the creatinine oxidase have not yet been made.

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The Effect of Denaturation upon the Sulfur Content of Egg Albumin and Edestin*

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INTRODUCTION

In 1938 we (1) showed that the nitrogen content of egg albumin and edestin is changed by certain types of denaturation. Acid and alkaline treatments were used as a means of denaturing the proteins. Edestin was also denatured by allowing it to stand in distilled water for a considerable time. It was found that the nitrogen contents of the proteins were lowered by these methods of denaturation, and the amount of nitrogen split off during the process of denaturation was insufficient to account for the observed lowering of the amount of nitrogen in the proteins. Long before this, Osborne (2) had found that the change of edestin to edestan lowered the percent of nitrogen to a considerable extent. Florence, Enselman, and Pozzi (3) in 1932, had also noted this decrease in percent of nitrogen to a considerable extent. Recently Bailey (4) has confirmed these findings.

In view of these results, we were interested to study the change in sulfur content brought about by certain forms of denaturation.

It is a well known fact, that in egg albumin solutions which have been treated with alkali and then acidified, hydrogen sulfide may be detected by the odor and by the lead acetate test. Treatment of egg albumin with acid also liberates a smaller but definite amount of hydrogen sulfide. Hopkins (5) in 1900 suggested that the volatile sulfide is liberated when egg albumin is denatured by shaking solutions of this protein. Quantitative data showing the change in sulfur content

* The data presented in this paper were taken from a thesis presented to the Graduate School of The University of Texas, June 1942, by Dr. Joe Dennis, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

of proteins on denaturation is not readily available, except that Osborne (6) has shown that edestan contains slightly greater percent of sulfur than edestin. He gives 0.833% for the sulfur content of edestin and 0.970% for edestan. It will be shown that our work confirms these findings, at least in a general way.

If the material split from the protein molecule by such denaturations is richer in sulfur than the protein itself, its removal should result in a diminution of the percentage of sulfur. The purpose of this present study is to determine whether the acid and alkali denaturation of egg albumin and edestin results in any significant quantitative alteration in their sulfur content, and also to determine whether the sulfur split from the protein is sufficient to account for the observed changes. In order that experimental errors may be ruled out in so far as possible, rather extensive analytical data have been gathered and the results have been compared by certain statistical methods.

METHODS

The usual methods for preparing egg albumin involve the use of sulfates and obviously are to be avoided, if possible, in a study such as this one. The following procedure was adopted for the preparation of native albumin. The whites from fresh hen eggs were saturated with sodium chloride and allowed to stand overnight for the flocculation of the globulins. The globulins were then filtered off, and a saturated solution of potassium dihydrogen phosphate was added to the filtrate until the reaction was distinctly green to bromocresol green. The egg albumin which precipitated was filtered off, dialyzed until practically free from chlorides and phosphates, and the solution was evaporated to dryness at room temperature.

It may be necessary to say something about the egg albumin prepared in this way. It is true that it did not crystallize, yet it has the physical properties of crystallized egg albumin, such as solubility in water and coagulability by heat. The sulfur content of this egg albumin as shown by the average of 49 determinations was found to be 1.645%, which, to be sure, is slightly higher than that reported by Osborne, who found 1.616%. However, our result is not significantly higher than some of the results which Osborne (6) reported from literature as having been determined by Hammersten as 1.64%, by Bondzynski and Zoya 1.66%, and by Kruger as 1.66%; it is however rather high as compared to the results of Hopkins, who found 1.57% of sulfur. If we may average our results with those of Osborne's and those which he quoted from the literature, we obtain a value of 1.630%. This average is only slightly lower than our results. The nitrogen content of egg albumin prepared by this method was found to be $15.34 \pm 0.04\%$, while Dennis (7) found $15.39 \pm 0.07\%$ for crystallized egg albumin. The median value of Dennis' 217 determinations was

15.35%. The average of all results which we have found reported in the literature for the nitrogen content of egg albumin, gives $15.35\% \pm 0.11\%$. We may conclude, therefore, that the albumin which was prepared by precipitation with KH_2PO_4 from sodium chloride solution does not differ significantly in nitrogen and sulfur content from that crystallized from sulfate solutions.

The acid denatured albumins were prepared by treating 30 g. samples of the native protein with 1800 ml. of acid at room temperature. The strength of acid employed was usually 0.5 *N* and the time of treatment twenty-four hours. The alkali denatured albumins were prepared by treating 30 g. samples of the native protein with 3 liters of 0.1 *N* sodium hydroxide for four hours at room temperature. The acid and alkali denatured albumins were precipitated by adjusting the reaction of the solutions to the point of maximum precipitation, using 1 *N* sodium hydroxide in the one case and 1 *N* hydrochloric acid in the other. The insoluble proteins were filtered off, washed thoroughly with water, and dried in air. The filtrates and washings from each preparation were combined, made alkaline, and evaporated to dryness. The proteins which were obtained by denaturation of egg albumin with acid or alkali were quite similar to what are usually called metaproteins. The yields from egg albumin varied from 95% in the case of one alkali denaturation experiment to 86.5% in the case of one in which acid denaturation was used, the yields in other experiments with egg albumin varied between these extremes.

The edestin was prepared according to the method of Osborne (2). The acid denatured edestin was prepared by treating 40 g. of the protein with 2 liters of 0.15 *N* hydrochloric acid for twenty-four hours at room temperature. The alkali denatured edestins were prepared by treating 40 g. samples of the native edestin with 2 liters of sodium hydroxide for four hours at room temperature. The strength of alkali employed was 0.2 *N* in one case and 0.5 *N* in the other. The denatured proteins were precipitated by adjusting the reaction of the solutions to the isoelectric point. The precipitated proteins were washed and the filtrates and washings handled in the same way as those from the denatured albumins. The proteins which were obtained by the denaturation of edestin with acid or alkali were quite obviously what are usually called metaproteins. The yields from edestin varied from 97.5% in acid denaturation to 73% in one case of alkali denaturation.

The moisture contents of the samples and the residue were determined by drying aliquots to constant weight in a vacuum oven at temperature of 105°C. and pressure of 45 mm. of mercury. The ash contents were determined by weighing after incineration at a temperature below 600°C. Sulfur contents were determined by sodium peroxide fusion followed by gravimetric determination of the sulfate as barium sulfate.

RESULTS

Fig. 1 shows clearly the difference in sulfur content between the native egg albumin and the acid denatured albumin. The average of forty-

nine determinations of sulfur in the native egg albumin gave $1.645\% \pm 0.019\%$ sulfur while sixty determinations of sulfur in acid denatured egg albumin gave $1.518 \pm 0.024\%$. Fig. 1 gives the suggestion that the acid denatured egg albumin was not entirely uniform because there is a second distinct peak to the histogram at approximately 1.623% sulfur

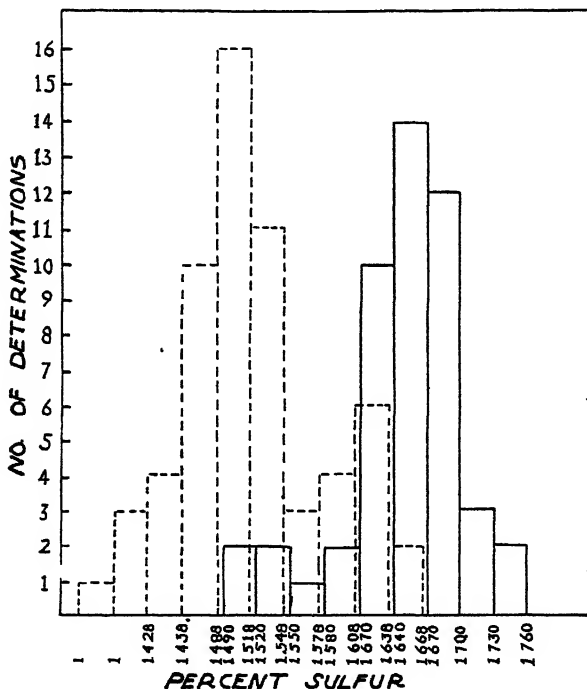


FIG. 1

Histograms Comparing Native Albumin With Acid Denatured Albumin
The denatured albumin is shown with broken lines and the native albumin with continuous lines

while the main peak is at approximately 1.503% sulfur. If we had chosen to discard the results on one of our preparations, the histogram for the acid denatured egg albumin would have been as symmetrical as could have been expected for such a small number of determinations and the peak would have been essentially the same as the main peak as it appears in Fig. 1. Our analyses of native egg albumin were done on two different preparations. The character of the histograms of these results

shows these preparations of egg albumin to be essentially identical because the histogram is as symmetrical as could be expected from such a small number of values. This fact is worthy of notice because our preparations of egg albumin were made by a method which is not ordinarily used and did not give a crystalline product.

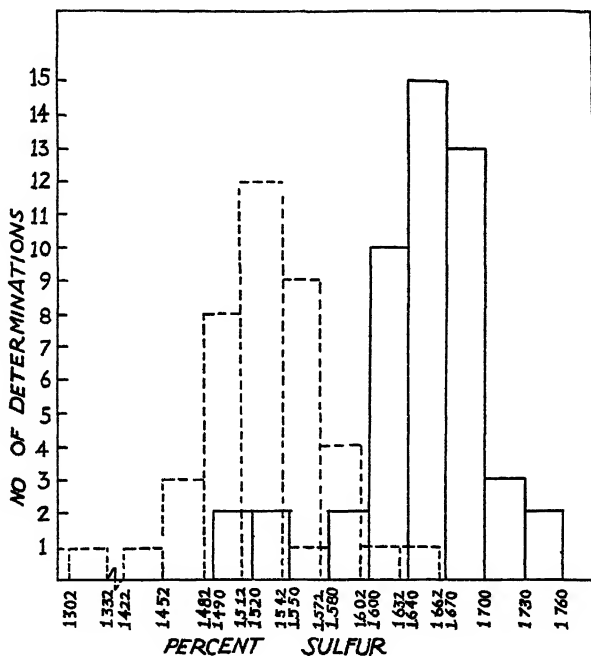


FIG. 2

Histograms Comparing Native Albumin With Alkali Denatured Albumin
The denatured albumin is shown with broken lines and the native albumin with continuous lines

Fig. 2 shows that the alkali denatured egg albumin contains less sulfur than the native egg albumin. The average amount of sulfur found was $1.527 \pm 0.018\%$. The distribution of the results as shown by the histogram in Fig. 2 is considerably more uniform than in case of the acid denatured egg albumin in spite of the fact that about half of these determinations were made on one preparation and half on another. The peak of the histogram is at approximately the same value as the arithmetical mean of all the results, which fact gives some slight support to the claim of uniform distribution of the results.

Fig. 3 shows the results of our comparison of the sulfur content of native edestin and alkali denatured edestin. The average sulfur content of the native edestin was $0.901 \pm 0.018\%$, twenty determinations having been made, while the average of thirty-one determinations of sulfur on the alkali denatured edestin was $0.850 \pm 0.011\%$. The histograms in Fig. 3 do not present as convincing a picture as those in Figs. 1 and 2, but we

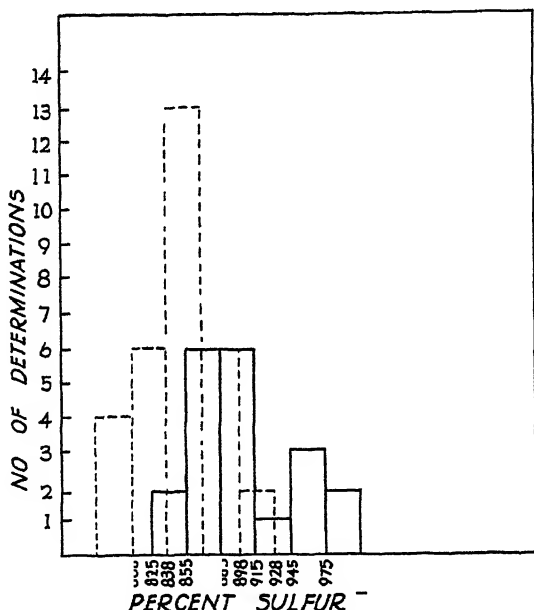


FIG. 3

Histograms Comparing Native Edestin With Alkali Denatured Edestin
The native protein is shown with continuous lines and the denatured protein with broken lines

will show later that there is a significant difference between the sulfur content of the native and alkali denatured edestin. It is worth while to call attention to the fact that the sulfur determinations on the alkali denatured edestin were made on two different preparations of the denatured protein showing further how uniform carefully controlled alkali denaturation can be, at least with respect to the sulfur content. The results on edestin and the alkali denatured edestin would have been much more convincing if as many determinations had been made on these pro-

teins as were made on egg albumin and its acid and alkali denatured products.

Fig. 4 compares the results of sulfur determinations on acid denatured edestin with those on the native edestin. These results are quite different from those given in the preceding figures. The denatured protein contains more sulfur than the native edestin, the average of twenty-one determinations being $0.934 \pm 0.017\%$. Fig. 4 does not show at a glance that there is a significant difference in the sulfur content, between the

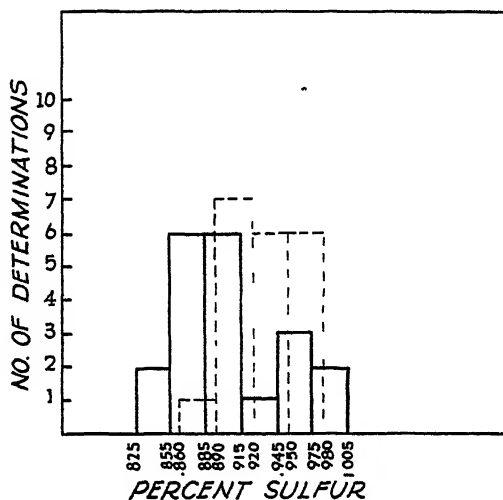


FIG. 4

Histograms Comparing Native Edestin With Acid Denatured Edestin
The denatured protein is shown with broken lines and the native protein with continuous lines

acid denatured edestin and the native protein, but it will be shown that there is a significant difference. It should be pointed out that acid denaturation of edestin did not split from the protein any sulfur containing organic substance, while every other case of denaturation described in this paper caused a loss from the protein of organic materials which are relatively rich in sulfur. A certain amount of organic substance was split from edestin during the process of denaturation by acid. It might be thought that this was merely the removal of some soluble impurity. Earlier work (1) has shown that nitrogen is split from edestin when it is changed to edestan by standing in contact with distilled water and also

that the resulting edestan has a lower percentage of nitrogen than the native protein. As mentioned above, Osborne (6) found more sulfur in edestan than in edestin. Suggestion has been made that this increase in sulfur might be due to some sulfur impurities in the reagents used in bringing about the change of edestin to edestan. In our acid denaturation of edestin, we have analyzed the resulting denatured protein, as well as the filtrate from the denaturing process. After the acid denaturation of 40 g. of edestin 3.66 g. of organic matter were found in the filtrate, but this same filtrate contained no sulfur. If the increase in sulfur content of the denatured protein had been due to sulfur impurities in the reagents, there surely would have been some sulfur containing compound in the filtrate for there is no evidence that sulfate ions are completely absorbed by proteins under conditions of fairly strong acidification with hydrochloric acid, such as existed in our preparations. There is no more reason to suppose that acid denatured edestin will absorb sulfate ions from solution than acid denatured egg albumin. We believe, therefore, that acid denaturing of edestin is essentially the same process as the action of the acid upon egg albumin differing only in the nature of the fraction split from the protein.

A further and more quantitative test of the significance of differences between the native and denatured protein is to be found in the *t* values as calculated according to Fisher (8). The *t* values are as follows:

Native egg albumin and alkali denatured egg albumin.....	<i>t</i> = 10.5
Native egg albumin and acid denatured egg albumin.....	<i>t</i> = 11.1
Native edestin and alkali denatured edestin.....	<i>t</i> = 4.16
Native edestin and acid denatured edestin.....	<i>t</i> = 2.91

These results show the probability that a real difference exists between the sulfur content of the native and denatured protein. This difference is so great that it may be regarded as definitely established.

In our earlier paper (1) we have shown that the nitrogen in the denatured protein plus the nitrogen in the filtrate did not balance with the nitrogen in the native protein. We suggested that water may have been taken up by the protein during the process of denaturation and calculated the number of molecules of water which, if added to the protein molecule, would bring about the observed changes in nitrogen content. Similar evidence for the addition of water during the process of denaturation has been found in connection with the study of the sulfur content of denatured proteins.

The following balance sheet of the acid denaturation of one sample of

egg albumin shows how evidence was obtained for the addition of water to the protein molecules:

Weight of moisture and ash free native protein used.....	27.29 g.
Sulfur in native protein.....	1.64%
Weight of sulfur in native protein.....	0.4464 g.
Weight of residue from filtrate evaporated.....	67.00 g.
Organic material in residue.....	3.84%
Weight of organic material in residue....	2.536 g.
Sulfur in organic material of residue.....	2.72%
Weight of sulfur in residue.....	0.0699 g.
Calculated weight of organic material in denatured protein	

$$(27.29 - 2.57) = 24.72$$

Calculated percent sulfur in acid denatured albumin

$$\frac{0.4464 - 0.0699}{24.72} \times 100 = 1.52$$

The observed percent of sulfur was 1.48%.

This and other similar results can be explained only by assuming that water was added during denaturation. By taking into account the molecular weights of protein, it can be shown from our data that 28 molecules of water were taken up by the acid denatured egg albumin, 15 by the alkali denatured egg albumin and 121 by the alkali denatured edestin. These, of course, are given only as suggestions of the amount of water added to the denatured proteins.

CONCLUSIONS

1. Acid and alkali denaturation of egg albumin result in a lowering of the sulfur content of this protein. Sulfur rich materials are split out of the protein by these treatments, and the denaturations appear to be accompanied by an addition of water to the protein.

2. Alkali denaturation of edestin resembles the acid and alkali denaturations of albumin.

3. Acid denaturation of edestin is different from the other acid and alkali denaturations studied in that no sulfur is removed from the protein.

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Determination of Glucuronic Acid in Biological Media*

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INTRODUCTION

In the method described by Maughan, Evelyn, and Browne (1) glucuronic acid in urine is determined by measuring the blue-violet compound formed by the reaction of glucuronides with naphthoresorcinol. Tollens (2) first proposed in 1908 the use of the naphthoresorcinol reaction; however, its specificity was questioned by Mandel and Neuberg (3). The limitations of Tollen's reagent were summarized by van der Haar (4). Salt (5) attempted to apply the reaction in the urine but was not successful. Kapp (6) by regulating the conditions under which the reaction occurs devised a micromethod for uronic acids. The method as modified by Maughan, *et al.* (1) has been found satisfactory by various workers (7, 8).

EXPERIMENTAL

In observations on glucuronic acid output in the urine after sulfonamide ingestion (9) an attempt was made to correlate sulfonamide concentration with glucuronic acid concentration in the blood. After testing various blood filtrates, it became apparent that the Maughan, *et al.* method would lend itself to estimating glucuronic acid there. The protein precipitating agents for blood which were tested included trichloroacetic acid, para toluene sulfonic acid, tungstic-sulfuric acid, zinc sulfate-sodium hydroxide, and copper sulfate-sodium tungstate. Of these, tungstic-sulfuric acid reagent yields the filtrate best suited for this purpose. Ether, petroleum ether, benzene, dioxane, chloroform were tested for their ability to extract the blue-violet compound, and only ether was satisfactory.

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Reagents. 10 per cent sodium tungstate solution,
2/3 *N* sulfuric acid solution,
0.2 per cent naphthoresorcinol solution.¹

This reagent is prepared by adding 0.1 g. of naphthoresorcinol to 50 ml. of distilled water, then placing this solution in the incubator at 37°C. for 24 hours. The solution is then filtered (the filtrate should be a clear brown) and kept in a dark colored bottle in the refrigerator. Enough should be prepared to last for only one week. A previous preparation should be saved to check each newly prepared reagent against a standard glucurone solution.

Merck's absolute anhydrous ether is washed with 1 per cent solution of ferrous sulfate, followed with several portions of distilled water, and finally dried over sodium sulfate. The complete removal of the oxidizing agent and traces of ferrous sulfate (wash reagent) is essential.

Removal of Proteins

Measure into a tube or flask 1 ml. of oxalated or citrated blood. Add slowly 7 ml. of distilled water, 1 ml. of 10 per cent sodium tungstate solution and 1 ml. of 2/3 *N* sulfuric acid. After 5 to 10 minutes filter. Two ml. of the filtrate, 2 ml. of 0.2 per cent solution of naphthoresorcinol and 2 ml. of concentrated hydrochloric acid are mixed in a test tube. The tube is heated in a boiling water bath for 45 minutes. At the end of this time remove the tube from the water bath and place in an ice bath for 10 minutes. Now add 2 ml. of 95 per cent ethyl alcohol and 15 ml. of prepared ether. Cork the tube (a glass stoppered tube is preferable) and shake vigorously for 30 to 60 seconds. Allow the ether layer which has a blue-violet color to separate. By inserting a pipette beneath the colored ether layer, the straw yellow or greenish colored solution may be removed and discarded. The colored ether solution is now transferred to a cuvette or a photo-electric colorimeter test tube and its light transmission measured. A filter transmitting light in the region of 565 μ is used. A blank and a control are analyzed simultaneously. The control employed was usually a 5 mg.-per cent of pure glucurone.²

¹ The naphthoresorcinol used in our experiments was obtained from B. L. Lemke, New York, Schwarz Laboratories, Inc., New York, and Eastman Kodak, Rochester, New York.

² We are indebted to Dr. G. J. Martin of the Warner Institute for Therapeutic Research and Dr. Walther Goebel of the Rockefeller Institute for the generous supplies of glucurone.

Biological fluids whose content of glucuronic acid fall in the range of 5 to 25 milligrams per 100 ml. may be treated in a like manner. If the expected glucuronic acid is more concentrated, the specimen is diluted to bring it within range. In dealing with biological fluids where proteins are present in negligible amounts, analyses may be made either directly or on a water dilution. Table I represents values of glucuronic acid obtained in cerebrospinal fluid, chest pus, and chest fluid.

TABLE I
Glucuronic Acid Concentrations in Spinal and Pleural Fluids and in Pus Cells

Specimen No.	Type of Fluid	mg./100 ml.
1	Spinal	1.2
2	"	0.5
3	"	2.0
4	"	1.4
5	"	1.3
6	"	2.1
7	"	1.5
8	"	2.6
9	"	2.0
10	"	2.3
11	Pleural	7.0
12	"	18.0
13	"	11.0
14	Pus Cells	8.2
15	"	7.9
16	"	8.3
17	"	6.5

Preparation of Curve

Fig. 1 indicates the relation of concentration to galvanometer readings and is obtained by preparing aqueous solutions wherein 2 ml. contain 10 to 60 γ glucurone. These are transferred into test tubes, and the tests are performed as outlined under method.

Sensitivity of Reaction

1, 2, 3, 4, and 5 mg. of glucurone per 100 ml. water were treated as indicated above. Two ml. of a 1/10 dilution of these solutions taken for analysis contain from 0.002 mg. (2 γ) to 0.01 mg. (10 γ). Concentrations up to 5 mg./100 ml. resulted, in color formations closely correspond-

ing to that of the blank. Thus when dealing with quantities less than 5 mg./100 ml. a dilution of 1:10 yields results out of accuracy range.

Time of Reaction

To ascertain the time of heating best suited for the reaction, pure glucurone solutions and different blood specimens were heated for various periods.

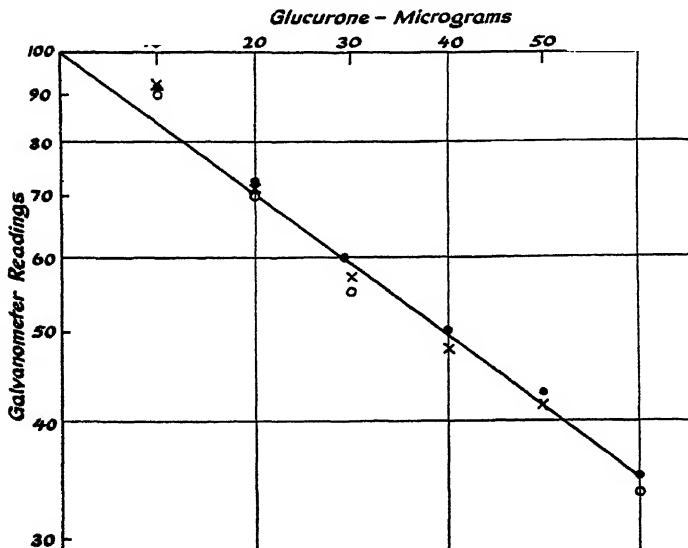


FIG. 1

Calibration Curve for Glucurone Made with a Photoelectric Colorimeter and Filter Transmitting Light in the Region of 565μ

Two ml. containing 10, 20, 30, and 50 γ of pure glucurone were heated for 30, 45, 60, and 90 minutes (Table II). The 45 minutes heating gave the best color extraction with ether.

A series of 50 blood specimens were prepared resulting in a ten-fold dilution, 2 ml. of each were heated for 30, 45, 60, and 90 minutes. Here too, the best color was obtained in the test tubes that were heated in the boiling water bath for 45 minutes. The average for the 30 minutes period was 5.6 mg. per 100 ml. of blood; for 45 minutes 8.6 mg. per 100 ml., for 60 minutes 8.0 mg. per 100 ml., and for 90 minutes 3.5 mg. per 100 ml. The most efficient period for heating appears to be 45 minutes. This time was consequently chosen as the standard period for heating.

TABLE II
Effect of Time of Heating upon Glucurone Recovery

Initial mg. Glucurone per 100 ml. of Solution	Mg. Glucurone per 100 ml. Solution Recovered	Time of Heating minutes	Recovery per cent
mg.	mg.		
5	3.5	30	70.0
	4.7	45	94.0
	4.0	60	80.0
	3.3	90	66.0
10	8.5	30	85.0
	11.2	45	112.0
	8.3	60	83.0
	7.0	90	70.0
15	13.8	30	92.0
	14.5	45	96.6
	17.0	60	113.3
	11.5	90	76.6
25	22.0	30	88.0
	24.4	45	97.6
	21.5	60	86.0
	12.7	90	50.8

TABLE III
Recovery Experiments

Recovery of Glucuronic Acid Added to Previously Analysed Bloods

Original Content of Glucuronic Acid mg./100 ml.	Calculated Glucuronic Acid mg./100 ml.	Quantity Found mg./100 ml.	Difference between Calculated and Found	Error per cent
6.8	8.0	9.7	1.7	21.2
6.0	7.5	7.9	0.4	5.3
6.8	8.0	7.0	-1.0	-11.0
7.5	8.4	9.9	1.5	17.8
6.0	7.5	8.5	1.0	14.0
7.0	8.1	8.1	0.0	0.0
5.5	7.2	7.6	0.4	6.2
5.0	6.9	7.2	0.5	7.2
4.5	6.6	6.7	0.15	2.3
6.5	17.3	20.0	2.7	15.6
11.5	21.7	21.7	0.0	0.0
7.0	17.5	18.5	1.0	5.5
5.5	15.0	17.1	1.3	8.5

TABLE IV

Milligrams Glucuronic Acid Determined Simultaneously with Milligrams Glucose per 100 ml. of Blood in Apparently Normal Individuals

Glucuronic Acid <i>mg./100 ml. of Blood</i>	Glucose <i>mg. 100 ml. of Blood</i>	Glucuronic Acid <i>mg./100 ml. of Blood</i>	Glucose <i>mg./100 ml. of Blood</i>
5.0	96.8	6.8	
5.0	177.5	6.8	80.0
5.0	97.0	7.0	277.0
5.0	105.0	7.0	
5.0	66.0	7.0	64.0
5.0	84.5	7.0	92.0
5.0	92.5	7.0	102.5
5.0	80.0	7.0	70.0
5.1	87.0	7.5	80.0
5.5	89.5	7.5	85.0
5.5		8.0	90.0
6.0	112.0	8.0	95.0
6.0	112.0	8.0	190.0
6.0	75.5	8.0	190.0
6.0	122.0	8.5	175.0
6.2	111.0	8.5	140.0
6.5	222.0	8.5	
6.5	123.0	8.5	
6.5	120.0	9.0	83.0
6.5	102.0	9.2	
6.5	100.0		

TABLE V

Glucuronic Acid Values for Blood of Diabetic Patients

Subject	Glucuronic Acid <i>mg./100 ml. of Blood</i>	Glucose <i>mg./100 ml. of Blood</i>
1	4.5	155.0
2	5.0	177.0
3	5.5	460.0
4	5.5	230.0
5	5.5	233.0
6	6.5	222.0
7	7.0	277.0
8	7.7	185.0
9	8.0	190.0
10	8.0	265.0
11	8.3	330.0
12	8.4	411.0
13	8.6	305.0
14	9.0	392.0
15	10.5	190.0
16	11.0	589.0

Recovery

A series of bloods were tested for recovery in which known amounts of glucurone were added to samples following previous analyses. These experiments are summarized in Table III. The per cent error ranged from -11 to 21.2

TABLE VI

Glucuronic Acid Values for Blood before and 24 hours after Sulfonamide Administration (Approx. 9 g.)

Subject	Sulfadiazine		Sulfathiazole	
	Before	After	Before	After
No.	mg./100 ml. of Blood	mg./100 ml. of Blood	mg./100 ml. of Blood	mg./100 ml. of Blood
1	5.9		7.3	
	5.0		7.0	
	6.0			8.0
	5.1			14.5
		12.5		8.5
		9.7		
		11.0		
2	10.5 ⁴		7.0	
		12.0		7.4
3	13.0		6.8	
		17.0		7.4
4	6.0	9.0		
5	6.0	9.0		
6	11.0	13.0		
7	6.0	9.5		
8	13.0	16.5		

Interfering Substances

Substances in the blood which might give this test are present only in small quantities and in the final dilution do not seem to interfere with the method. Pentoses do not interfere because the color which arises with naphthoresorcinol is insoluble in ether. Using the tungstic-sulfuric acid filtrate, glucose estimations (10) were carried out simultaneously

with the determination of glucuronic acid. In a series of 110 analyses, the average concentration of glucuronic acid in the blood was 7.5 mg. per 100 ml. of blood. Table IV indicates some glucuronic acid and glucose values obtained with apparently normal individuals. The glucuronic acid concentrations vary independently of the glucose values. To further demonstrate the lack of glucose interference, pure solutions of glucose were subjected to glucuronic acid test. Fifty, 100, 150, and 200 mg.-per cent of glucose solutions resulted in galvanometer readings corresponding closely to that of the blank. Glucuronic acid and glucose values obtained in a group of diabetic subjects are shown in Table V. The high glucose values did not influence normal glucuronic acid concentrations. A group of subjects were tested for glucuronic acid before and 24 hours after sulfonamide ingestion. The results are given in Table VI. Following sulfadiazine administration there appears to be a marked increase in the glucuronic acid in the blood.

SUMMARY

A method for the determination of glucuronic acid in blood and other biological fluids is described. The procedure is based on the Maughan, Evelyn, and Browne technique for determining glucuronic acid in urine. Normal values for glucuronic acid in blood are reported. There is an increase in glucuronic acid in the blood following sulfadiazine ingestion.

Miss Orneata M. Holder assisted in the determinations.

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Growth Promotion on Tryptophan-Deficient Media by *o*-Aminobenzoic Acid and Its Attempted Reversal with Orthanilamide

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INTRODUCTION

It was early shown (1) that anthranilic acid (*o*-aminobenzoic acid) was one product resulting from the degradative action of bacteria on tryptophan. This finding has been confirmed (2) and several intermediate stages in the conversion postulated. Recently, Kotake (3) showed that anthranilic acid is also formed from tryptophan by the rat. Attempts to substitute it for tryptophan in the rat diet failed, however, although a temporary cessation in weight loss did result. It therefore appeared that this degradation was essentially irreversible in the animal body.

A number of microorganisms, including the lactic acid bacteria (4), are known which require tryptophan for growth. Fildes (5) showed that indole replaces tryptophan for those strains of *B. typhosum* which require it, and postulated that tryptophan synthesis proceeded through the indole stage. The demonstration that anthranilic acid could replace tryptophan in its growth-promoting effect for certain microorganisms would provide a further possible intermediate for this biosynthesis. It would further provide an excellent opportunity to extend the known relationship between growth promotion by *p*-aminobenzoic acid and its inhibition by sulfanilamide (6) to entirely different, yet closely related compounds.

In the experiments described below, it was found that certain, but not all, species of lactic acid bacteria could utilize anthranilic acid in place of tryptophan. Growth thus produced was not inhibited by high concentrations of the corresponding sulfonic acid (orphanilic acid), its amide (orphanilamide) or by 2-(orphanilamido)-pyridine.

EXPERIMENTAL

The medium used throughout was similar to that described for detection and estimation of pseudopyridoxin (7), but contained no added tryptophan and was supplemented by addition of 3 γ pyridoxin hydrochloride per 10 cc. The cultures

TABLE I
*Anthranilic Acid and Indole as Substitutes for Tryptophan in Growth of Lactic Acid Bacteria**

Substance tested	Amount added γ /10 cc.	<i>L. arabinosus</i>	<i>L. pentosus</i>	<i>L. casei</i>	<i>S. lactis</i>	<i>L. mesenteroides</i>
—	0	3.0	12	4.0	4.0	2.0
Tryptophan	0.3	10	15	9.0	13	8.0
	1.0	23	20	20	24	22
	3.0	44	32	43	45	41
	10	67	54	63	54	60
	30	84	63	77	66	65
	100	90	63	80	64	65
Anthranilic Acid	0.3	10	12	8.0	4.0	2.0
	1.0	22	12	11	4.0	2.0
	3.0	35	—	15	—	—
	10	49	—	37	—	—
	30	59	12	45	4.0	2.0
	100	60	—	51	—	—
	1000	10	12	58	4.0	2.0
Indole	0.3	16	12	15	4.0	2.0
	1.0	36	12	31	4.0	2.0
	3.0	51	—	47	—	—
	10	60	—	52	—	—
	30	68	12	47	4.0	2.0
	100	76	—	50	—	—
	1000	82	12	43	4.0	2.0

* Incubation period: 24 hours. Zero reading of the galvanometer represents light transmission of distilled water; a reading of 100 is complete opacity.

used were *Lactobacillus arabinosus* 17-5; *Lactobacillus pentosus* 124-2; *Streptococcus lactis* R; *Lactobacillus casei*; and *Leuconostoc mesenteroides* P-60. *L. casei* was grown at 37°C.; other organisms were grown at 30°C. Inocula were grown for 24 hours in the above medium supplemented with 500 γ tryptophan per tube, then washed and diluted before use as described previously (7).

RESULTS

The growth response produced by addition of tryptophan, anthranilic acid, or indole to the deficient culture medium is shown in Table I.

Anthranilic acid supported growth of *L. arabinosus* and *L. casei* in the absence of tryptophan; it did not bring growth to the same high levels reached with tryptophan, however. At levels approaching 1 mg. per 10 cc. of medium it was consistently toxic for *L. arabinosus*; consequently at this high level no growth effect could be detected. It was not available for growth of *S. lactis*, *L. mesenteroides*, or *L. pentosus*. Each of the cultures which could utilize anthranilic acid for growth in place of tryptophan also utilized indole for this purpose; those which did not utilize anthranilic acid did not utilize indole. Separate experiments

TABLE II

Effect of Orthanilic Acid, Orthanilamide and 2-(Ortho-anilamido)-pyridine on the Growth-Promoting Action of Anthranilic Acid for Lactic Acid Bacteria

Substance tested	Amount added γ per 10 cc.	Amount of anthranilic acid present γ per 10 cc.	Galvanometer reading*	
			<i>L. arabinosus</i>	<i>L. casei</i>
—	—	0	4.0	2.0
—	—	1.0	22	14
—	—	30	49	50
Orthanilic acid	10,000	1.0	27†	17†
Orthanilamide	1,000	1.0	22	14
	3,000	1.0	22	14
	10,000	1.0	19	13
2-(Orthanilamido)-pyridine	1,000	1.0	22	13

* As in Table I.

† The slight increase in galvanometer reading was due to slight color imparted to the culture medium by the added orthanilic acid, and was not due to increased growth.

showed that salicylic acid had no growth-promoting action under these conditions. *Meta*- and *para*-aminobenzoic acids were likewise completely inactive.

An experiment was next designed to determine whether the growth-promoting effect of anthranilic acid was nullified by the presence of orthanilic acid (*o*-aminobenzenesulfonic acid), orthanilamide, or 2-(ortho-anilamido)-pyridine,* in analogy to the *p*-aminobenzoic acid-sulfanilamide relationship. Results are shown in Table II. Amounts of orthanilic acid and of orthanilamide 10,000 times as great as the amount of anthranilic acid present failed to suppress the growth-promoting

* We wish to thank Dr. R. O. Roblin, Jr., American Cyanamide Co., for samples of orthanilamide and 2-(orthanilamido)-pyridine.

action of anthranilic acid. Orthanilamide appeared to suppress growth very slightly when 10 mg. was added per 10 cc. of medium. Separate experiments showed the same slight inhibitory action at this level when tryptophan was used to promote growth instead of anthranilic acid; hence the inhibition cannot be due to structural similarity of the two compounds. At higher concentrations (30–100 mg. per tube) orthanilamide prevents growth completely even when excess tryptophan is present.

DISCUSSION

The results cited above show that for some organisms anthranilic acid exerts the same growth-promoting action on a tryptophan-deficient medium as does tryptophan. This is interpreted to mean that these organisms are capable of synthesizing tryptophan from anthranilic acid. The reverse reaction has already been demonstrated with both bacteria and animals. Organisms capable of utilizing anthranilic acid for this purpose can also utilize indole. The fact that organisms which cannot synthesize tryptophan from ammonia or from the amino acids available in casein hydrolyzates can do so from these two substances does not, of course, prove that these substances are intermediates in the synthesis of tryptophan in nature. Fildes (5) has already postulated, however, that tryptophan synthesis proceeds through indole, and it may well be that anthranilic acid also functions here. Indeed, it is quite possible that indole is further degraded to anthranilic acid before being built into tryptophan *via* other intermediates. Investigation of a large number of organisms might reveal some which could utilize indole, but not anthranilic acid, or *vice versa*, and thus indicate the direction which tryptophan synthesis takes in nature.

It was anticipated that the growth-promoting action of anthranilic acid would be reduced or nullified by addition of orthanilic acid or orthanilamide, because the structure of these substances is related to that of anthranilic acid in the same way that certain other inhibitors are to other growth essentials, *e.g.* *p*-aminobenzoic acid-sulfanilamide (6); pantothenic acid-pantoyl taurine (8, 9). This did not prove to be the case. It should be pointed out, however, that the highest level of these compounds which could be tested was only 10,000 times as great as the amount of anthranilic acid which was necessary to produce an easily measurable growth effect.

McIlwain (9) has defined the *antibacterial index* of growth inhibitors

of this type as C_I/C_M , where C_I is the concentration of an inhibitor just bacteriostatic in the presence of a concentration C_M of the corresponding metabolite. The antistreptococcal index of sulfanilamide (metabolite: *p*-aminobenzoic acid) is then 25,000; the antistaphylococcal index of pyridine-3-sulfonamide (metabolite: nicotinamide) is 250,000.

From the data here presented, it can only be said that the anti-bacterial index of orthanilamide (metabolite: anthranilic acid) for *L. arabinosus* and *L. casei* is greater than 10,000 and failure to obtain growth inhibition at the levels tested cannot be construed as evidence against the theory that inhibitors of the nature of sulfanilamide function because of structural similarities to certain essential metabolites. It is well known that different organisms vary greatly in their sensitivity to anti-bacterial agents; it is possible that among other organisms capable of utilizing anthranilic acid some might be found which would be inhibited by orthanilic acid or its amides, and that this inhibition would be alleviated by additional anthranilic acid. *L. arabinosus*, however, is very sensitive to antibacterial agents of this nature so far investigated. From data already available, it is possible to calculate the antibacterial indices of sulfanilamide (10) and pantoyl-taurine (8) for this organism. For sulfanilamide, the value is about 70; for pantoyl-taurine, about 350. Thus the antibacterial power of orthanilamide for this organism, if such exists, is of much lower order than in either of these two related cases.

SUMMARY

Anthranilic acid is capable of replacing tryptophan for *L. arabinosus* and for *L. casei*, but not for a number of other species of lactic acid bacteria. Those cultures capable of utilizing anthranilic acid can also utilize indole.

The growth-promoting action of anthranilic acid was not influenced by simultaneous additions of much higher levels of orthanilic acid, orthanilamide or 2-(orthanilamido)-pyridine. Implications of these results are discussed briefly.

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Inhibiting Action of Mannose upon the Growing Plant

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INTRODUCTION

The action of sugars upon the growing plant was investigated by L. Knudson (1) on various plants (corn, pea, vetch, cabbage, etc.) grown in nutrient solutions fortified by additional sugars examined. In these experiments, hexoses as well as the disaccharides investigated, caused increased plant growth. On the other hand, galactose has shown toxic effects even at concentrations as low as 0.0125 *M*, its toxicity being partially overcome by 0.05 *M* glucose.

In another series of experiments Knudson (2) investigated the action of sugars on plants grown in culture tubes on a nutrient agar medium under conditions insuring freedom from microorganisms. To a modified Pfeiffer's nutrient solution sugars were added at a concentration of 0.025 *M*. In these experiments galactose has shown toxic effects on the roots of peas and wheat. This toxicity was prevented by glucose or sucrose. In addition, mannose, at a concentration of 0.025 *M*, has been found to be very toxic to vetch, peas, and wheat. Its toxicity was overcome by either glucose or sucrose.

F. L. Wynd (3) studied the action of various sugars on orchid seedlings. The sugar was added to three inorganic media in amounts sufficient to give 7 g. of carbon per liter. The growth of the seedlings was greatest in seedlings grown in *d*-mannose solution, and was gradually lessened in seedlings grown in *d*-glucose, maltose, *d*-fructose, sucrose, or raffinose. No growth was obtained on *d*-galactose. In these experiments mannose proved the best nutrient addition for the growing orchid.

Ch. L. Schneider (4) has investigated the action of sugars on the growing plant with plant sections (*Avena coleoptile*) immersed in test solutions. The elongation of the sections as compared with controls was greatly increased in 0.2 and 1.0 per cent solutions of dextrose, levulose, or sucrose.

EXPERIMENTAL

In our experiments the action of various hexoses and hexitols on the

growth of the intact plant was studied on cress (*Cardamine*) seedlings germinated and grown in tap water only.

The germinated cress seedling is naturally provided with sufficient food for the first few days of its development, except for water which must be furnished from outside. Thus, it grows suitably in tap water only. Under these conditions, any change in the growth rate of the seedlings observed after the addition of the investigated substance to the tap water in which the seedlings are grown, really represents the effect of the added substance, and the probability of an interference from complicating metabolic processes due to the presence of additional substances in the nutrient medium can be excluded.

In our experiments the cress seedlings were germinated on moistened filter paper under strictly determined conditions. The details of the method used by us have been described by B. Lustig and H. Wachtel (5).

The cress seeds were obtained from P. Henderson & Co., 35 Cortland Street, New York City ("Extra curled cress, # 380"). The best seeds selected from the commercial mixture were used for the experiments.

Each experiment consisted of many tests, each of which included 50 selected seeds placed upon five layers of filter paper in a Petri dish of 7 cm. diameter. The filter paper was moistened with 6 cc. of water or the test solution. The Petri dishes were covered by glass tumblers, and kept at room temperature in a moderately illuminated location.

Under the above described conditions, the cress seeds germinate in less than 24 hours, and after a few days the seedlings develop in a perfectly uniform manner into plants of considerable size.

Each experiment included observation of 200-300 seedlings growing in 6 cc. of the test solution while an equal number of seedlings growing in 6 cc. of tap water served as comparison controls.

The hexoses dextrose, galactose, levulose, and mannose, and the hexitols manitol, sorbitol, and dulcitol, have been studied. C. P. substances were used. Hexoses were obtained from Eimer and Amend, N. Y., and hexitols from Pfanstiehl Chemical Co., Waukegan, Ill. Various amounts of the substances under investigation were added to 6 cc. of tap water and the effect of those solutions on the growth of the seedlings observed.

The results of our observations are summarized in the Table I. The numbers in Table I give in millimeters the average size of the seedling's stems as attained in the various solutions at the time the stems of the controls reached a size of 25 mm. The amount of the substances added to 6 cc. of water in which the seedlings were growing varied from 2 to 40 milligrams. Table I gives the values for each of the growth attainments at the given concentration. In view of the low aqueous solubility of dulcitol its highest concentrated test solution contained only 12 mg. in 6 cc. of water.

The experiments have shown that the more highly concentrated solutions of the substances (40 and 20 mg. in 6 cc. of water) produced marked inhibition of the stem growth of the seedlings as compared with the stem growth of the controls. The inhibition of the stem growth was especially noticeable in the mannose solutions, where the seedlings attained a stem growth of only 5 and 11 mm. by the time the stem growth of the controls reached a stem length of 25 mm. Solutions of levulose or sorbitol containing 12 mg. in 6 cc. of water produced a slight inhibition of stem growth while mannose solution of this same concentration caused a markedly higher growth inhibition. Less concentrated solutions of the substances (9, 6, and 4 mg. in 6 cc. of water) did not inhibit the stem growth

TABLE I

Average Length of Stem (in millimeters) of Seedlings Growing in Solutions of Hexoses and Hexitols

Substance used	Milligrams of substance present in 6 cc. of nutrient solution						
	40	20	12	9	6	4	2
Controls*.....	25	25	25	25	25	25	25
Dextrose.....	21	23	25	25	25	25	25
Galactose.....	16	23	25	25	25	25	25
Levulose.....	19	23	23	25	25	25	25
Mannose.....	5	11	18	20	20	23	25
Mannitol.....	20	22	25	25	25	25	25
Sorbitol.....	19	22	22	25	25	25	25
Dulcitol.....			25	25	25	25	25

* Controls were grown in 6 cc. of tap water.

more greatly, except mannose solutions which lost their growth inhibiting property at a concentration of 2 mg. in 6 cc. of water (0.0018 *M*).

The root growth of the seedlings was not changed by any of the substances examined except mannose. The seedlings growing in mannose solutions containing 40 mg. of the substance in 6 cc. of water did not develop roots at all; those growing in mannose solution containing 20 mg. in 6 cc. of water developed very short roots, in sharp contrast with the better developed, although short, stems of the seedlings. Solutions containing 12, 9, or 6 mg. of mannose in 6 cc. of water caused a slight inhibition of the root growth. This inhibition was not more present in seedlings growing in the solution of 4 mg. of mannose in 6 cc. of water (0.0037 *M*).

The inhibition of the stem growth observed in seedlings growing in the more highly concentrated solutions of the substances examined is caused

by the relatively high osmotic pressure of these solutions. The plant growth is influenced by the degree of osmotic pressure of the nutrient fluid. Control experiments have proved that solutions of growth-indifferent substances equimolar with the growth-inhibiting solutions of substances examined by us (excepting those of mannose) always cause

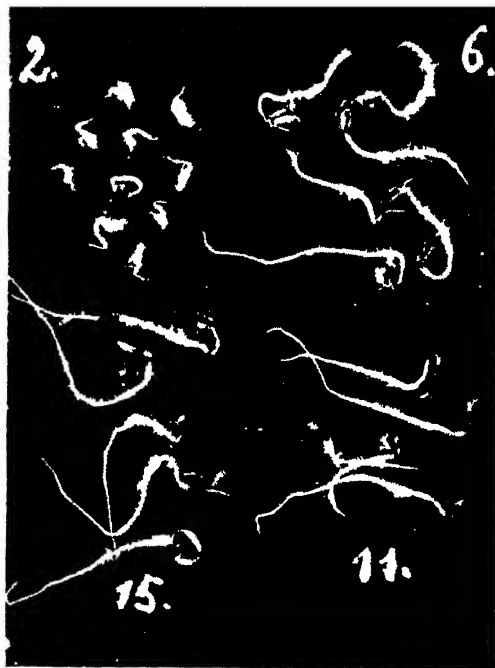


FIG. 1

Cardamine Seedlings Grown for Three Days in 0.1 *M* Solutions of: (2) Mannose; (6) Dextrose; (15) Levulose; (11) Galactose

The growth of the mannose seedlings is strongly inhibited

the same degree of inhibition of the stem growth of the seedlings. No growth inhibition occurs in solutions of growth-indifferent substances equimolar with the solution of 10 mg. of hexose in 6 cc. of water (0.009 *M*).

Thus, no specific growth inhibiting action has been found exhibited by dextrose, galactose, levulose, mannitol, sorbitol, or dulcitol solutions. On the other hand, a clearly marked inhibition of the growth of the seed-

lings was produced by mannose solutions. The growth inhibition, in the case of the stem as well as of the root, caused by mannose was stronger when larger amounts of the substance were present in the solutions in which the seedlings were growing.

In addition, our experiments have shown that mannose also inhibits germination of the cress seeds in a specific manner.

Table II summarizes the results of our experiments on the influence of substances on the germination of cress seeds. Table II gives the percentage of cress seeds germinated after 19 hours in the various solutions as referred to the number of germinated controls taken as 100%.

Solutions of substances which are 0.17 *M* or higher inhibit germination of the seeds regardless of the organic or inorganic nature of the substances (5). This inhibition of germination is related to the osmotic

TABLE II
Percentage of Cress Seeds Germinated, Referred to the Number of Controls Germinated as 100%

Substance used	Concentrations of the solutions (molarity)					
	0.415	0.37	0.33	0.17	0.125	0.1
Dextrose	zero	45	70	80	100	100
Galactose	zero	35	70	85	100	100
Levulose	zero	35	65	85	100	100
Mannose	zero	10	30	80	85	100
Mannitol			50	100	100	100
Sorbitol			70	100	100	100

pressure of the solutions and was also exhibited by our substances. In addition, mannose solutions caused inhibition of germination in concentrations as low as 0.125 *M*.

The results of our experiments, thus, show that mannose has a toxic growth-inhibiting action on the growing plant. This action is closely connected with the stereochemical structure of mannose. It is not present either in its alcohol, mannitol, nor in the other hexoses or hexitols examined.

DISCUSSION

Comparing the above results with the results of the investigations included in the literature, we note in our experiments the absence of any growth-inhibiting action of galactose, contrary to the data obtained by Knudson or Wynd. Considering the differences between our experimental method and that of the authors mentioned, the explanation of

the disparity of results is to be found in the circumstance that in our experiments no other substances were present in the nutrient medium except the sugar investigated. Galactose itself has no growth-inhibiting properties, and the observed growth-inhibition represents a secondary effect of products of galactose metabolism which, in the presence of other substances in the nutrient medium, give rise to new substances which unfavorably influence the growth of the seedlings.

In this connection, it must be noted that the addition of hexoses did not increase the growth rate of the seedlings in our experiments. We have investigated the growth activity of our substances in solutions as low as $0.000004 M$. A slight and variable growth-promoting action was sometimes found in dextrose solutions between 0.0006 and $0.0002 M$. The action was more apparent in the solutions of lower concentration. This growth promotion was insignificant as compared with the growth promotion effected by auxins and auxin-like substances. The effect must be thus ascribed to the nutrient value of dextrose. It is a further proof of the utility of the method used here that pure nutritive effects only appear to an insignificant extent.

On the other hand, minute amounts of hexitols in concentrations between 0.0003 and $0.00008 M$ (0.35 – 0.1 mg. in 6 cc. tap water) have produced a more marked and more constant growth-promoting action which was more apparent in mannitol and dulcitol, and weaker in sorbitol solutions. A slight growth-promoting action of hexitols must thus be surmised. Further experiments are necessary to clarify this question.

Our experiments confirm the growth inhibiting action of mannose. This peculiar behavior of mannose may be correlated with the experimental data indicating the possibility of ascorbic acid formation from mannose. This possibility was emphasized for the plant both *in vivo* and *in vitro* (6), and was also stated for animal tissues in partially controversial investigations by many authors (7).

Our experiments indicate that mannose plays a special role in the metabolism of the plant, and that this is different than the role of the other hexoses.

SUMMARY

Mannose added to the nutrient medium in which plant seedlings are growing inhibits the growth of the seedlings more highly when larger amounts of this substance are added. This action is connected with the

stereochemical structure of mannose, and is not present in solutions of dextrose, galactose, levulose, mannitol, sorbitol, or dulcitol.

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The Effect of Heavy Metals on the Activation and Injury of the Enzyme Tyrosinase*

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INTRODUCTION

Papers dealing with the toxicity of chemical elements for whole animals, isolated tissues, cells of various types, and many derivatives of biological material have appeared from time to time (1). Since the heavy metals are known to definitely affect proteins, much interest and speculation have arisen in attempts to explain such action. Certain of the heavy metal salts, notably HgCl_2 , have been used by cytologists as an essential constituent of fixatives for cells.

Data recently accumulated on the properties of the enzyme tyrosinase indicate its protein nature (2). They also seem to show that its activation is probably connected with some fundamental physicochemical change in the protein molecule. It becomes of some interest, therefore, to determine the reactions of protyrosinase and tyrosinase to salts of heavy metals. The present investigation was undertaken to determine the activating capacity as well as the toxicity of certain heavy metals for this enzyme.

MATERIALS AND METHODS

Protyrosinase was extracted from diapause grasshopper (*Melanoplus differentialis*) eggs according to a procedure previously described (3). Samples of the protyrosinase were standardized by activation with aerosol OT so as to catalyze the uptake of 100 cu. mm. of O_2 by 0.3 ml. of 0.4 per cent tyramine-HCl at 25°C. in 10 minutes. For convenience, this will be referred to as 100% activation of the protyrosinase.

The mixture in each vessel of the Warburg manometers consisted of the following: approximately 0.3 ml. of protyrosinase, 0.5 ml. of Sorensen's phosphate

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(0.2 *M* in respect to the phosphate) buffer (pH 6.8), 0.3 ml. of aerosol OT when used, the amount of the salt of the heavy metal to yield the desired concentration, 0.3 ml. of tyramine-HCl in the side bulb, and enough 0.9 per cent NaCl solution to make the total fluid volume in each vessel 3.00 ml. The manometers were shaken at a rate of 120 oscillations per minute through an amplitude of 2 cm.

The salts (C.P.) used were the following: $\text{AuCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{PdCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$, HgCl_2 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CdCl}_2 \cdot 2\text{H}_2\text{O}$, ZnCl_2 , MnCl_2 , AlCl_3 , and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$. In all cases they were dissolved in 0.9 per cent NaCl solution. The aerosol OT was dissolved in glass redistilled water since a 1%

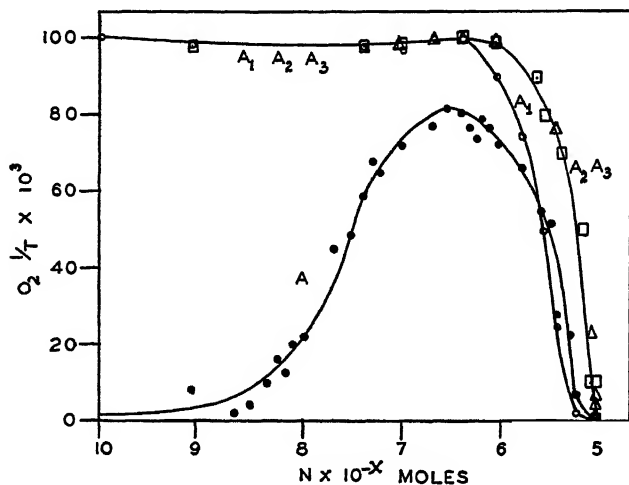


FIG. 1

Shows the Activation as well as Toxicity of HgCl_2 for Prottyrosinase and Tyrosinase

Abscissae = concentration of HgCl_2 in moles. Ordinates = rate of O_2 uptake in terms of aerosol OT activated prottyrosinase taken as 100%. A = prottyrosinase treated with HgCl_2 alone. A₁ = Addition of aerosol OT to HgCl_2 treated prottyrosinase. A₂ and A₃ = effect of adding HgCl_2 to tyrosinase obtained by activation with temperature (Δ) or aerosol OT (\square).

solution can not be obtained in 0.9 per cent NaCl solution at room temperature. All quantities of the salts of heavy metals referred to are the actual number of moles acting on a given amount of prottyrosinase in a total fluid volume of 3.0 ml.

RESULTS

The action of 1×10^{-10} to 1×10^{-8} moles of HgCl_2 on prottyrosinase is an activation effect (Fig. 1A). Concentrations of HgCl_2 above 1×10^{-8} increasingly activate prottyrosinase until at 3×10^{-7} moles there is an activation of 82% in relation to aerosol OT activation of a similar

amount of protyrosinase. Concentrations between 3×10^{-7} and 1×10^{-5} moles are toxic and at the latter concentration tyrosinase is irreversibly injured (Fig. 1A).

When protyrosinase is treated for ten minutes with activating concentrations of HgCl_2 and aerosol OT is then added 100% activation occurs (Fig. 1A₁). If protyrosinase is converted into tyrosinase either by aéro-

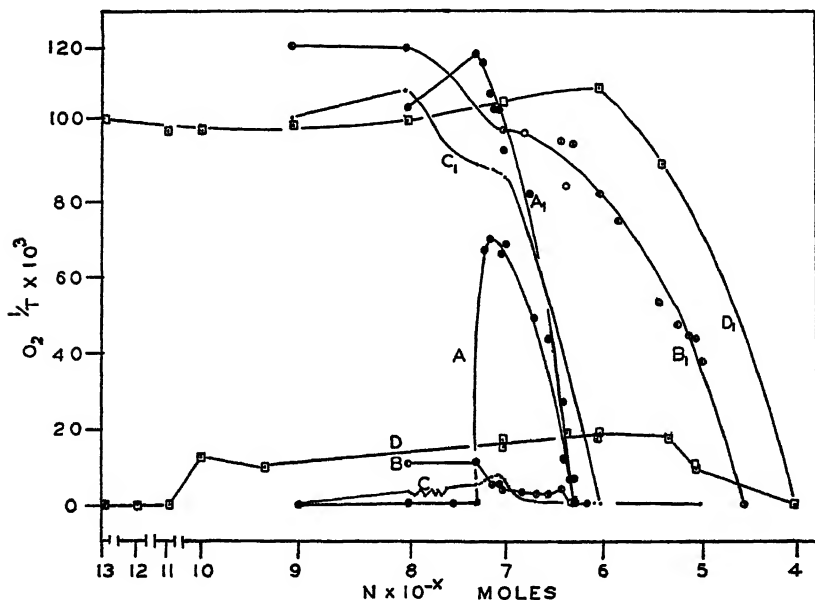


FIG. 2
Same as Fig. 1

A = protyrosinase treated with AuCl_3 . A₁ = addition of aerosol OT to AuCl_3 treated protyrosinase. B = same as A for H_2PtCl_6 . B₁ = same as A₁ for H_2PtCl_6 . C = same as A for PdCl_2 . C₁ = same as A₁ for PdCl_2 . D = same as A for CuCl_2 . D₁ = same as A₁ for CuCl_2 .

sol OT (4) or by heat activation (5) and then to it activating concentrations of HgCl_2 are added, 100% activity is always obtained (Figs. 1A₂ and A₃). Tyrosinase obtained either by heat or by aerosol OT activation seems to be more resistant to toxic concentrations of HgCl_2 than tyrosinase produced by treatment with HgCl_2 alone (compare Figs. 1A, A₂, and A₃).

Concentrations of AuCl_3 up to 5×10^{-5} moles do not activate protyro-

sinase (Fig. 2A). Activation occurs abruptly at 6×10^{-8} moles reaching a maximum of 70% at 7×10^{-8} moles. At a concentration of 5×10^{-7} moles complete inactivity is present. The addition of aerosol OT to AuCl_3 -treated protyrosinase in subactivating concentrations yields

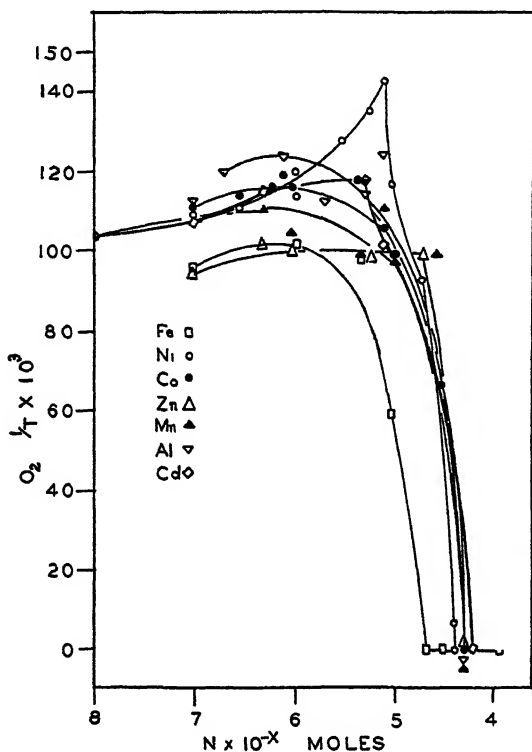


FIG. 3

Shows Effect of Adding Aerosol OT to Tyrosinase Treated with Toxic Concentration of Various Salts
Otherwise same as Fig. 1

complete activation (Fig. 2A₁). Addition of aerosol OT to activating concentrations produces a marked overshooting of the 100% value (Fig. 2A₁). Toxic concentrations of AuCl_3 plus aerosol OT show a slightly decreased toxicity similar to that noted for HgCl_2 + aerosol OT.

The maximum protyrosinase activation by H_2PtCl_6 amounts to approximately 10% between concentrations of 1×10^{-8} and 6×10^{-8}

moles (Fig. 2B). At higher concentrations the activity gradually drops and reaches zero at 5×10^{-7} moles. The addition of aerosol OT to protyrosinase treated with activating concentrations of Pt yields approximately 120% activation (Fig. 2B₁). The activity reaches zero at 3×10^{-5} moles.

Palladium activation begins at 2×10^{-9} and reaches an 8% maximum at approximately 8×10^{-8} moles (Fig. 2C). Complete destruction of the enzyme occurs at 3×10^{-7} moles. The action of Pd plus aerosol OT is similar to Pt plus aerosol OT except the overshooting is not as great (Fig. 2C₁).

CuCl₂ has a wide range of activation between 1×10^{-11} and 5×10^{-6} moles (Fig. 2D). The enzyme is completely destroyed at 1×10^{-4} moles. At the optimum concentration of approximately 1×10^{-6} moles Cu activates the protyrosinase 20%. Cu treated protyrosinase upon the addition of aerosol OT yields practically 100% activation with but a slight tendency for overshooting (Fig. 2D₁).

FeCl₃, NiCl₂, CoCl₂, CdCl₂, ZnCl₂, MnCl₂, and AlCl₃ do not activate protyrosinase between concentrations of 1×10^{-8} and 1×10^{-4} moles (Fig. 3). When protyrosinase is subjected to these salts within concentrations of 1×10^{-8} and 5×10^{-6} moles for ten minutes and to them aerosol OT is added 100% or more activity is obtained. A marked tendency for overshooting is present reaching a maximum in case of Ni of 143.5% (see Fig. 3). At higher concentrations these metals are toxic to tyrosinase.

DISCUSSION

Heavy metals that appreciably activate protyrosinase yield activation curves which have maxima at definite concentrations (Figs. 1 and 2). Their ranges of activating concentrations vary to a rather extreme degree, being highest and most pronounced in the case of HgCl₂ and CuCl₂ (Figs. 1 and 2). For HgCl₂ we have values ranging from 1×10^{-10} to 3×10^{-7} moles. Within these concentrations there seems to be no injury to the enzyme preparation since upon the addition of aerosol OT complete activation occurs. However, it should be noted that for HgCl₂ alone some 18% inhibition in activity at the maximum activating concentrations occurs. The exact nature of this inhibition is at present obscure, but that it is in some way associated with the activating mechanism is indicated by the following. Protyrosinase, converted to tyrosinase, either by aerosol OT or by heat when treated with activating concentra-

tions of HgCl_2 shows no inhibiting effects of the salt. That this is not the result of a primary action of the HgCl_2 on the aerosol OT or substrate can be readily shown by experimental combinations of the various reagents. All evidence at hand seems, therefore, to indicate an inhibiting action of the HgCl_2 on the protyrosinase as such or on the protyrosinase molecule during the process of conversion to the tyrosinase or active state. The change of the inactive to active condition (protyrosinase to tyrosinase) apparently is an extremely sensitive and complex phenomenon as far as the action of HgCl_2 is concerned. Further evidence along these lines may be gained from a study of the manner in which "toxic" concentrations of HgCl_2 affect the enzyme preparation.

Beyond the maximum activating concentrations of HgCl_2 toxicity is definitely present as indicated in Curve A of Fig. 1. Protyrosinase treated with such concentrations of HgCl_2 reacts in a typical and uniformly toxic manner and eventually a concentration is reached at which the enzyme is completely destroyed (Fig. 1). Results of treatment with concentrations in the low toxicity range for HgCl_2 alone when compared with those for similar enzyme preparations treated in slightly different fashions seem further to indicate a rather complex action of the "activating" salts. Addition of aerosol OT to enzyme preparations treated with low toxic concentrations of HgCl_2 (1×10^{-7} to 3×10^{-6} moles) decreases the apparent toxicity of the HgCl_2 (Fig. 1A₁). After these concentrations are reached (3×10^{-6} moles) the addition of aerosol OT has no further effect (Fig. 1A₁). That this "toxic decreasing effect of aerosol OT for HgCl_2 " is a complex one can be further shown in another manner. If tyrosinase, produced by either aerosol OT or heat activation, is treated with known toxic concentrations of HgCl_2 the toxicity is further decreased as shown in Fig. 1A₂ and A₃. In this experiment we begin with the active enzyme, tyrosinase alone, so that the effects of the toxic concentrations of HgCl_2 in this instance must be solely upon the tyrosinase. The difference in reaction in this type of experiment as compared with that described above, indicates a marked and fundamental dissimilarity in the action of HgCl_2 on tyrosinase as such and the "protyrosinase to tyrosinase" conditions as produced by HgCl_2 alone. At the present time we have no explanation as to the exact site of action of the HgCl_2 but merely wish to emphasize the extreme complexity of such reactions.

The range of the AuCl_3 activating curve is very narrow (Fig. 2A) in comparison to the Hg curve. The addition of aerosol OT to the Au

treated enzyme between concentrations of 1×10^{-8} and 2×10^{-7} not only yields 100% activation but also overshoots approximately 19% at a concentration of 5×10^{-8} moles (Fig. 2A₁). At present we have no explanation for this phenomenon. As with Hg, Au also at a sharply defined concentration produces complete inactivation of the enzyme (Fig. 2A and A₁). Whether the activation of the enzyme is accomplished

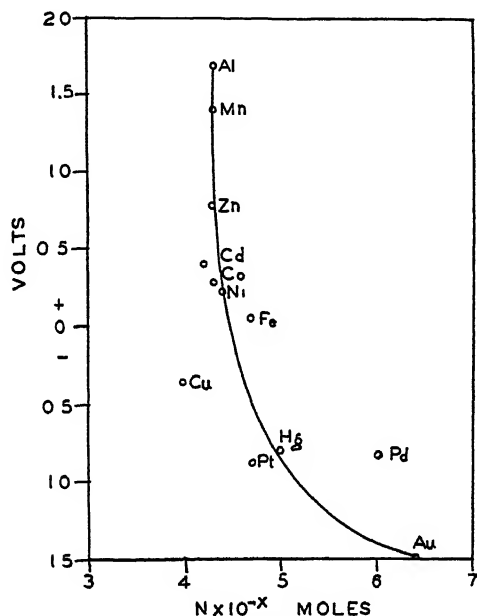


Fig. 4

Shows Relation of Toxicity of Heavy Metal Cations for Tyrosinase and their Position in the Electromotive Series
Abscissae, same as Fig. 1. Ordinates, potential in volts

with Au alone or with Au and aerosol OT the concentration of Au necessary for 100% destruction is the same. In this respect it will be noted that Au is similar to Hg.

Platinum (Fig. 2B) and palladium (Fig. 2C) salts activate protyrosinase very slightly. After the action of these salts the addition of aerosol OT also overshoots the 100% activity (Fig. 2B₁ and C₁). These are the only salts whose points of complete inactivation with and without aerosol do not coincide.

Copper activates protyrosinase approximately 20% but it has a very wide range of activity (Fig. 2D). It is the least toxic of all the salts used. This may be due to the fact that the enzyme contains copper in the prosthetic group (6). Previously it was shown (3) that copper can be taken out of the prosthetic group of the enzyme and later replaced by inorganic copper. It could not be substituted by Fe, Co, Ni, Mn, and Zn. Experimentally it seems clear that none of these cations can replace copper. The overshooting, therefore, is doubtless not caused by the replacement of the copper in the prosthetic group. It may be pointed out that gold not only activates but also overshoots. None of these salts exerts a catalytic action for the oxidation of tyramine-HCl alone since experimental results obtained are entirely negative.

The toxicity of the 12 elements placed in the descending order is: Au > Pd > Hg > Fe > Pt > Ni > Co > Zn, Mn, Al > Cd > Cu. A comparison of this group with the electromotive series of metals is presented in Fig. 4. There seems to be a possible relation in the toxicity of an ion and its position in the electromotive series. It should be pointed out, however, that the toxicity of the cations is independent of the valence of the ion, of the atomic number, and of the grouping in the periodic table. Jones (7) showed that the toxicity of the elements on the platyhelminth *Policelis nigra* was related to the solution tension of the ions.

Only those metals lower in the electromotive series than copper activate protyrosinase. Although the toxicity of the cations on tyrosinase may be related to the solution tension of the ions concerned, the activation of protyrosinase does not depend on the electromotive series either in concentration of the salt necessary or in the percentage of activity brought about by the activating salts.

SUMMARY AND CONCLUSIONS

1. The effects of salts of the heavy metals—mercury, gold, palladium, iron, platinum, nickel, cobalt, zinc, manganese, aluminum, cadmium and copper—on the activation and toxicity for protyrosinase and tyrosinase have been studied.

2. Activation of protyrosinase occurs within definite concentration of Hg, Au, Pt, and Pd.

3. Below toxic concentrations of all salts no injury to the protyrosinase was evidenced since the addition of a known activator of protyrosinase, aerosol OT, produced 100% activation.

4. All salts in higher concentrations are toxic for tyrosinase, the order of toxicity being, $\text{Au} > \text{Pd} > \text{Hg} > \text{Fe} > \text{Pt} > \text{Ni} > \text{Co} > \text{Zn}, \text{Mn}, \text{Al} > \text{Cd} > \text{Cu}$.

5. The possible relations of the toxicity of the salts and their position in the electromotive series are discussed.

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The Denaturation of Tobacco Mosaic Virus by Urea

I. Biochemical Aspects

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I. INTRODUCTION

In a preliminary communication (1) it was pointed out that tobacco mosaic virus, TMV, is denatured when it is dissolved in 6 *M* urea buffered to about pH 7. It was found that the action of urea on the virus resulted in a decrease in specific infectivity, in the appearance of free sulfhydryl groups, in a separation of nucleic acid, in a loss of solubility in dilute neutral salt solutions, and in a decrease in average particle size or molecular weight. The authors were directed to an investigation of these matters by a report of Frampton and Saum (2) in which it was claimed that TMV is dispersed by concentrated urea into fragments with a molecular weight of about 10^6 and that this change takes place without loss of infectivity. The consequences of this claim were so far-reaching that it was deemed advisable to study the action of urea on TMV in considerable detail. As reported in the preliminary communication (1), the authors were able to verify the disintegrating action of urea on the virus but were unable to find any conditions under which this disintegration could proceed without the complete destruction of infectivity. In the meantime, Martin (3) also reported on the disintegrating action of urea, and Bawden and Pirie (4), in a rather comprehensive study, failed to obtain any evidence that marked chemical and physical changes in the virus nucleoprotein could take place without causing inactivation, thereby confirming the authors' contentions. In this paper the biochemical experiments carried out in our laboratory which pertain to this matter are described in some detail. Experiments on the kinetic aspects of this question are described elsewhere (5).

II. EXPERIMENTAL RESULTS

A. Solubility Changes

Normal TMV is soluble in dilute solutions of neutral electrolytes. The solubility characteristics of the urea-denatured virus, however, are entirely different. The degradation products are quite soluble in 6 *M* or stronger urea solutions, but, as the urea concentration is reduced by dilution with neutral electrolyte solutions, the material precipitates. The solubility is considerably less in 4.5 *M* urea and is very low in 3 *M* urea. No detectable amount remains in solution when the urea concentration is reduced to 0.6 *M*. This precipitated protein material can be redissolved in 6 *M* urea solutions. It dissolves quite readily in very dilute solutions of a synthetic detergent, "Orvus," or of sodium dodecyl sulfate. As much as or more than 30 or 40 mg. of the denatured protein can be dissolved in 1 cc. of a 0.1 per cent "Orvus" solution. The precipitated protein will also dissolve readily in 0.1 *M* sodium hydroxide, but not at all readily in 0.01 *M* sodium hydroxide. Comparable experiments carried out on the coagulum obtained upon heat denaturation of the virus indicate that the solubility of this material is similar to that of the urea-denatured material.

B. The Reduction in Molecular Weight

1. *Osmotic Pressure Studies.* A solution containing 11 mg. of TMV per cc. in 6 *M* urea and 0.1 *M* phosphate buffer at about pH 7 was placed in a stationary osmometer with a cellophane diaphragm and equilibrated against the solvent. The temperature was maintained at 24.6°C. The results of this, a typical experiment, are shown in Table I. Since TMV has a molecular weight of around 4×10^7 and a negligible osmotic pressure, the results in the table demonstrate that the urea denaturation of the virus is accompanied by a tremendous decrease in molecular weight.

2. *High-speed Quantity Centrifugation.* Normal TMV can be sedimented quantitatively from its solutions when spun for 1 hour at 30,000 r.p.m. in a Bauer-Pickels (6) high-speed quantity centrifuge. In 16 experiments in which various concentrations of different electrolytes were used, it was observed that the longer the virus was allowed to remain in contact with 6 *M* urea before centrifugation, the smaller was the amount of protein which could be separated by such treatment. The kind and concentration of electrolytes used are described in Table I of Reference 5. In a representative experiment, a 20 cc. portion of a solu-

tion containing about 10 mg. of virus per cc. in 6 *M* urea and 0.12 *M* sodium chloride was allowed to stand at room temperature. At the end of 1, 2, 4, and 8 days, 5 cc. aliquots were centrifuged for 1½ hours at 30,000 r.p.m. The pellets and the supernatant fluids were analyzed for protein nitrogen. The results are shown in Table II. These and the comparable data obtained in the other 15 experiments can be interpreted to indicate a gradual decrease in the amount of high molecular weight material.

TABLE I

Increase in the Osmotic Pressure Due to the Action of 6 M Urea on TMV

Time*	Pressure	Av. Mol. Wt.	Time	Pressure	Av. Mol. Wt.
days	mm. water		days	mm. water	
0	0		8	48	55,000
1	2	1,400,000	10	50	52,000
2	7.5	350,000	12	52	50,000
3	10	270,000	16	55	48,000
4	17	155,000	18	61	43,000
5	28	93,000	22	63	42,000
6	35	75,000	28	64	41,000

* The differences between the rates of disintegration of TMV which could be calculated from the data of this table and those of Tables II, III, IV, and V do not indicate that the reaction rates differ for different criteria of denaturation. At the time these experiments were begun, the extreme sensitivity of the rates of urea denaturation of TMV to such variables as temperature, pH, ionic strength, and initial virus concentration was not realized, and no attempt was made to fix the conditions of reaction exactly. For example, systems described in this paper as being at about pH 7 could actually have been as much as 0.2 or 0.3 pH units above or below that value. Carefully executed experiments on the kinetics of this process are described elsewhere (5).

3. *Ultracentrifugation Experiments.* A sample of TMV was allowed to denature in 6 *M* urea and 0.1 *M* phosphate buffer at pH 7. The reaction products were precipitated by dilution of the urea solution and were isolated by centrifugation at about 3,000 r.p.m. After several washings with water, a portion of this precipitate was dissolved in a 0.1 per cent solution of "Orvus" and the remainder in a 6 *M* urea solution. These solutions were then studied in the analytical ultracentrifuge. The sedimenting boundaries were followed by the Svensson (7) modification of the schlieren method. At a protein concentration of 3.2 per cent, the material dissolved in "Orvus" had a sedimentation constant of 3.1×10^{-13} and that dissolved in urea had a constant of 2.8×10^{-13} cm. per sec.

per unit field. The boundaries resembled those of fairly homogeneous proteins. In another experiment, TMV was dissolved in 6 *M* urea and 0.1 *M* phosphate buffer at about pH 7. After 5 hours, and again after 29 hours, this solution was examined in the ultracentrifuge. Single boundaries were observed with sedimentation constants of 1.56 and 1.66×10^{-13} , respectively. In still another experiment, the degradation products were isolated as described above and dissolved in 0.5 per cent sodium dodecyl sulfate and .025 *M* sodium acetate-0.1 *M* sodium chloride buffer at pH 5.5. In this case the sedimentation constant was found to be about 8.2×10^{-13} . Since no detailed information concerning the shape of the particles of these degradation products is available, it is not possible to calculate accurate molecular weights from these data. Unless the particles are exceedingly unsymmetrical, one can conclude, however, that the fragments obtained when urea acts upon TMV may be small enough

TABLE II

Decrease in the Amount of High Molecular Weight Protein Due to the Action of 6 M Urea on TMV

Time days	Milligrams protein per cc. of original solution	
	<i>in pellet</i>	<i>in supernatant</i>
1	8.17	0.95
2	7.70	2.25
4	3.44	4.76
8	0.19	8.96

to have average molecular weights as low as 10 or 15 thousand or may be large enough to have molecular weights between 200 and 300 thousand, depending upon conditions which at present are not sufficiently understood. Particles of comparable size were found when heat-denatured virus protein dissolved in 6 *M* urea or in 0.1 *M* sodium hydroxide was examined in the ultracentrifuge.

4. *Decrease in Stream Double Refraction.* Takahashi and Rawlins (8) first showed that TMV solutions exhibit double refraction of flow. The authors demonstrated that the intensity of this flow double refraction could be measured by the deflection of a galvanometer coupled into a suitable photoelectric circuit (9). A solution containing 10 mg. per cc. of TMV in 6 *M* urea and 0.1 *M* phosphate buffer at about pH 7 was studied in the double refraction apparatus at room temperature. In Table III, the galvanometer deflections are shown as a function of time of standing. It may be seen that 6 *M* urea gradually destroys the ability

of TMV to exhibit double refraction of flow. This result may be interpreted as being due to the destruction of the very large and very anisometric rod-like virus particles which are responsible for the strong stream double refraction. Mehl (10) has also observed the decrease in stream double refraction of TMV due to the action of urea. Bawden and Pirie (4) used this change as an indication of virus nucleoprotein destruction in their study on the effects of urea on viruses.

TABLE III

Decrease in Stream Double Refraction Due to the Action of 6 M Urea on TMV

Time of standing	Galvanometer deflection (intensity of stream double refraction)
<i>hours</i>	<i>mm.</i>
0.10	27.8
0.50	24.3
1.00	17.6
2.00	10.5
4.00	5.0
7.75	1.1

TABLE IV

Decrease in Turbidity Due to the Action of 6 M Urea on TMV

Time	Colorimeter	Time	Colorimeter
<i>minutes</i>	reading	<i>minutes</i>	reading
0	85.0	106	41.8
6	77.0	138	36.3
14	72.0	179	29.3
23	66.3	201	27.0
34	62.5	255	23.3
54	56.0	319	21.0
79.5	48.0		

5. *Decrease in Turbidity.* Solutions of TMV in water are opalescent. This is due to the fact that the virus particles are large enough to scatter light and anisometric enough to give the appearance of sheen. Immediately following solution of the virus in 6 M urea, the system is opalescent, but upon standing this opalescence gradually disappears until finally a perfectly clear solution is obtained. The decrease in opalescence can be followed quantitatively with a photoelectric colorimeter of the Klett-Summerson type. In Table IV may be seen the results of a typical experiment in which a solution containing 9 mg. per cc. of TMV in 6 M urea and 0.1 M phosphate buffer at about pH 7 was allowed to stand at

25°C. The colorimeter is so constructed that the intensity of color or turbidity is directly proportional to the reading. This result provides additional evidence of the disintegrating action of urea on TMV.

6. *Correlation of Molecular Weight Studies.* The results obtained by means of high-speed quantity centrifugation, stream double refraction studies, and turbidimetric studies all agree in indicating at the qualitative level that normal TMV is broken down into much smaller fragments by the action of 6 *M* urea. The results of the osmotic pressure study show that these fragments may have molecular weight as low as 40,000. In agreement with this result, the ultracentrifugation studies indicated that the molecular weight may be as low as 10 or 15 thousand or as high as 200 or 300 thousand, depending upon the conditions under which the degradation is carried out. Frampton and Saum (2) concluded from their diffusion results that the molecular weight of the "virus" in urea is of the order of magnitude of 10^5 , and Martin (3) concluded from sedimentation studies that the molecular weight of the urea degradation products of the virus is about 400,000. There is, then, complete agreement that, under the proper conditions, strong urea solutions cause a degradation of TMV into smaller particles, and there is no serious discrepancy between the estimates of the size of the urea degradation products obtained by various methods and by several investigators.

C. *The Loss of Nucleic Acid*

A solution containing 250 mg. of TMV, 1.16 g. of boric acid, 0.48 g. of sodium tetraborate, and 9.01 g. of urea in a total volume of 25 cc. was prepared and allowed to stand at room temperature. At the end of two and four hours, 2 cc. samples of the material were diluted to 10 cc. with 0.2 *M* sodium chloride. The resulting precipitates were isolated by centrifugation and were washed twice in 0.2 *M* sodium chloride solution, then dissolved in dilute sodium hydroxide and analyzed for nitrogen by the Kjeldahl method and for phosphorus by the King (11) method. The precipitates were found to contain 1.03 and 1.73 mg. of nitrogen, respectively, and no measurable amounts of phosphorus. Since the nitrogen to phosphorus ratio for normal TMV is about 30, the King method is sufficiently sensitive to measure accurately the phosphorus in a virus sample containing 0.5 mg. of nitrogen. Therefore, the results demonstrate that, when TMV is allowed to stand in strong urea solutions, the protein gradually loses most if not all of its phosphorus. This may be interpreted to mean that nucleic acid is split off when the virus is de-

natured in strong urea. This result was mentioned in the preliminary announcement of the authors (1) and was confirmed by Bawden and Pirie (4). The latter found, in addition, that nucleic acid is also liberated when potato virus X is denatured by urea but that it is not liberated by tomato bushy stunt and tobacco necrosis viruses when subjected to comparable treatment. Nucleic acid is liberated when TMV is denatured by heat (12, 13) and by high pressures (14).

D. Appearance of Sulfhydryl Groups

Tobacco mosaic virus contains an amount of cysteine sulfur corresponding to 0.7 per cent cysteine (15, 16); yet solutions of the active virus do not give a pink color with nitroprusside and are not oxidized by ferricyanide or porphyrindin; hence, the cysteine sulfhydryl groups are not free to react with these reagents. However, following denaturation of the virus in 6 *M* urea, the mixture was found to give a pink color with nitroprusside, indicative of the presence of free sulfhydryl groups. The number of these groups was estimated with porphyrindin according to the method described by Greenstein (17). In a typical experiment, the titration of a solution prepared by adding 2 g. of urea to 2 cc. of a solution containing 73 mg. of TMV at pH 8 corresponded to 0.70 per cent cysteine. In another experiment in which 3.2 g. of guanidine hydrochloride were used instead of urea, the titration of the solution at pH 7 corresponded to 0.76 per cent cysteine. Similar results were obtained when the sulfhydryl groups were estimated in the presence of 6 *M* urea or guanidine by means of ferricyanide, tetrathionate, or mercuribenzoate (18). These findings indicate that, following denaturation in concentrated urea or guanidine, all or most of the sulfur of TMV becomes free and measurable as sulfhydryl sulfur.

E. Reduction of Infectivity

As was shown in section B, 6, there is complete agreement among the several workers who have studied this question that TMV is broken down into particles with molecular weights of a few hundred thousand or less when it is brought in contact with strong solutions of urea. It is of the utmost importance to ascertain whether these small fragments possess virus infectivity, as claimed by Frampton and Saum (2). Several experiments designed to answer this question are described in the following paragraphs.

The sample of TMV in urea used in the stream double refraction

experiment described in section B. 4, was tested periodically for infectivity by rubbing certain dilutions of the material and of an untreated virus solution on opposite halves of 30 or more leaves of *Nicotiana glutinosa* L. It may be seen from the results shown in Table V that virus infectivity is gradually decreased by the action of urea and that, under the particular conditions of this experiment, the reduction is more than 99.9 per cent in 96 hours. This gradual decrease in virus infectivity by the action of urea was confirmed by dozens of experiments in our own laboratory and by a great many experiments reported by Bawden and Pirie (4).

It was next necessary to determine whether the infectivity remaining after the denaturation is partially completed is associated with the rod-

TABLE V
Decrease in Infectivity Due to the Action of 6 M Urea on TMV

Hours	Virus in urea		Control		Estimate of per cent reduction of infectivity
	g. protein cc. in inoculum	No. of lesions per half leaf	No. of lesions per half leaf	g. protein cc. in inoculum	
0.1	10 ⁻⁵	42.7	38.3	5 × 10 ⁻⁶	< 50
	10 ⁻⁴	103.9	46.2	10 ⁻⁵	
0.5	10 ⁻⁴	73.4	36.8	10 ⁻⁵	> 50
1	10 ⁻⁴	60.9	41.2	10 ⁻⁵	ca 75
2	10 ⁻⁴	55.2	17.7	10 ⁻⁶	> 90
4	10 ⁻⁴	37.5	10.5	10 ⁻⁶	> 90
8	10 ⁻⁴	12.4	7.8	10 ⁻⁶	ca 99
30	10 ⁻⁴	2.9	0.9	10 ⁻⁷	> 99
96	10 ⁻⁴	0.24	1.91	10 ⁻⁷	> 99.9

shaped, high molecular weight nucleoprotein particles or with the low molecular weight degradation products. In order to answer this question, ten 6 M urea solutions containing about 10 mg. of TMV per cc., differing from each other only in the amount and kind of electrolyte present, were allowed to stand at room temperature. The compositions of these solutions are listed in Table I of Reference 5. At the end of 24, 48, 96, and 192 hours, 5 cc. samples of each were centrifuged for 1½ hours at 30,000 r.p.m. The fluid in the upper third of each tube was pipetted off carefully and analyzed for protein nitrogen. Portions of each original solution and of each supernatant fluid were each diluted 1 to 100 with 0.1 M phosphate buffer and were tested for virus infectivity on *Nicotiana glutinosa* plants. Ten whole leaves were used for the original solutions and 5 for the supernatant fluids. This type of infectivity test is much

less accurate than the half-leaf test usually used in this laboratory, but it is entirely adequate for the detection of large differences in virus activity. The results of this study are shown in Table VI. These data show conclusively that virus infectivity is associated with the high molecular weight, easily sedimentable nucleoprotein and not with the low molecular weight urea degradation products.

TABLE VI

Distribution of Infectivity between High and Low Molecular Weight Components Following the Treatment of TMV with 6 M Urea

Expt. No.	End of 24 hours			End of 48 hours			End of 96 hours			End of 192 hours		
	Lesions/leaf		Protein supern.	Lesions/leaf		Protein supern.	Lesions/leaf		Protein supern.	Lesions/leaf		Protein supern.
	Orig. fluid	Supern. fluid		Orig. fluid	Supern. fluid		Orig. fluid	Supern. fluid		Orig. fluid	Supern. fluid	
			mg./cc.						mg./cc.			
1*	64.8	0.2	0.61	17.6†	0.2	1.26	60.8	0.0	1.36	20	0.0	5.18
2	43.2	0.0	0.85	22.6†	0.2	1.50	36.5	0.0	3.54	6.45	0.0	8.44
4	70.6	0.2	1.12	26.8†	0.6, 0.0†	2.21	80.1	0.0	5.20	14.5	0.0	9.06
6	70.6	0.0	0.10	12.6†	0.6, 0.0†	0.10	159.9	0.2	0.54	60.3	0.0	0.85
7	6.5	0.0	8.62	3.0	0.0	8.83	2.5	0.0	8.21	0.4	0.0	8.74
9	0.7	0.0	8.48	0.1	0.0	8.54	0.9	0.0	8.50	0.2	0.0	9.36
11	35.2	0.0		67.6	0.0		106.3	0.0	0.01	131.9	0.0	0.10
12	70.8	0.0	4.28	9.10†	0.6, 0.0†	5.04	38.7	0.0	5.72	3.65	0.0	8.92
14	2.0	0.0	8.40	0.3	0.2	8.58	1.75	0.0	9.10	0.1	0.0	8.54
16	46.8	0.0	0.71	43.6	0.2	4.40	61.4	0.0	5.60	32.1	0.0	6.90

* Experiment numbers correspond to those of Table I of Reference 5, where the compositions of the solutions are listed.

† Diluted 1 to 1000 instead of 1 to 100.

‡ Retested.

In one experiment, a portion of the supernatant fluid obtained upon the high-speed centrifugation of a partially denatured virus sample was dialyzed for 43 hours against distilled water, in order to remove urea slowly. The original supernatant fluid and the dialyzate were both diluted 1 to 100 with 0.1 M phosphate buffer and each was inoculated to 14 or more whole leaves of *Nicotiana glutinosa*. No lesions were obtained in either case. Hence, the process which changes high molecular weight,

active virus into low molecular weight, inactive fragments cannot be reversed by the simple means of removing urea slowly by dialysis.

In order to determine whether the inactivation of the virus was a direct result of the complete disintegration of the nucleoprotein molecule, the pellets obtained after centrifugation for $1\frac{1}{2}$ hours at 30,000 r.p.m. of 3 of the original solutions described in Table VI were tested for infectivity by comparison with untreated virus from the stock sample. The relatively accurate half-leaf method was employed. Thirty-two leaves of *Nicotiana glutinosa* were used in each test. The pellet obtained at the end of 96 hours from Sample 4 and that obtained at the end of 192 hours from Sample 11 of Table VI were diluted with 0.1 *M* phosphate buffer at pH 7 to protein concentrations of 2×10^{-6} g./cc. The pellet obtained at the end of 96 hours from Sample 12 was diluted to a concentration of 4×10^{-6} . These were compared with the untreated virus solution at a concentration of 10^{-6} g./cc. The fractions, 351/64, 466/210, and 664/308, respectively, represent the total number of lesions given by the control divided by the number given by the treated virus for each case. It is evident that the untreated virus solution is more infectious than any of the more concentrated treated solutions. These data indicate that most of the virus particles lose their infectivity before extensive disintegration occurs in concentrated urea. Hence, urea denaturation of TMV would seem to involve at least two consecutive reactions.

III. DISCUSSION

The experiments reported in this paper demonstrate that TMV nucleoprotein is denatured by the action of 6 *M* urea. The denatured material is soluble in 6 *M* or stronger urea, in certain synthetic detergents, and in dilute alkali, but is insoluble in dilute neutral salt solutions. The molecular weight of this material is much lower than that of the intact virus—of the order of magnitude of 10^4 or 10^5 . During the denaturation process, nucleic acid is separated from the protein and the sulfur of the virus becomes free and measurable as sulfhydryl sulfur. Most important of all, virus activity is lost even before the nucleoprotein disintegrates extensively, and the degradation products are completely void of infectivity. Bawden and Pirie (4) have confirmed many of these observations and have made the additional observations that the urea-denatured material is serologically quite distinct from the intact virus and that many aspects of this behavior of TMV in urea are common to potato virus X, tobacco necrosis virus, and tomato bushy stunt virus. The rela-

tionship of the denaturation of viruses in urea to the behavior of other proteins under comparable conditions is discussed in some detail by Bawden and Pirie (4).

The divergence between the points of view of Frampton and of the authors remains to be considered. Frampton seems to regard TMV solutions as being made up of colloidal macro-aggregates of very small fundamental virus molecules. The action of urea, he appears to believe, is to disperse these aggregates into the component molecules, thereby reducing the anomaly in the viscosity and increasing the magnitude of the diffusion constant. Denaturation is admitted by Frampton (19) to occur, but he seems to feel that this takes place only after a long time, while the other changes are essentially instantaneous. To a certain extent, the views of the authors are compatible with those of Frampton. Indirect evidence that TMV nucleoprotein particles with a molecular weight of about 4×10^7 can aggregate end-to-end to form dimers, trimers, etc., has been presented by Bawden and Pirie (12) and by the authors (20). Recently, direct confirmation of this aggregation process was obtained by means of electron microscopy (21). One of the actions of urea on TMV might well be the reversal of this aggregation process, and that reversal might well proceed faster than denaturation. Regardless of the mechanism by which one explains anomalous viscosity, such a dispersion should decrease it. Beyond this point, however, the authors disagree with Frampton. Frampton and Saum (2) concluded that the infectious unit of TMV has a molecular weight of the order of 10^5 . In view of the extensive series of experiments reported in the present paper in which it was found that virus infectivity remaining after exposure to urea was always associated with material which could be removed by centrifugation for $1\frac{1}{2}$ hours at 30,000 r.p.m., the authors hold to their original belief that the infectious unit of tobacco mosaic virus is a particle with a molecular weight of about 4×10^7 , and that material obtained from urea solutions with a molecular weight of around 10^5 is nothing more than a virus degradation product possessing no virus activity. The conclusion of Frampton and Saum (2) that virus activity is associated with low molecular weight products obtained from TMV upon solution in 6 *M* urea has not been confirmed.

IV. SUMMARY

The behavior of tobacco mosaic virus in essentially neutral 6 *M* solutions of urea was studied. It was found that the virus was transformed

from a material soluble in dilute aqueous electrolyte solutions to one insoluble in such solvents. These changes were shown by means of osmotic pressure, high-speed quantity centrifugation, ultracentrifugation, stream double refraction, and turbidimetric studies, to be accompanied by a disintegration of the high molecular weight virus nucleoprotein particles into much smaller fragments. Phosphorus analyses indicated that the nucleic acid was split away from the protein in this disintegration. The number of measurable sulfhydryl groups was found to increase during denaturation. The action of urea on the virus was also demonstrated to result in a loss of virus infectivity. Not only was it shown that residual infectivity was always associated with remaining high molecular weight nucleoprotein in cases of partial denaturation, but it was also shown that the specific infectivity of such residual material was considerably less than that of untreated virus, indicating that virus inactivation is a reaction which may take place before the virus nucleoprotein molecule is extensively disintegrated. No means of reversing the over-all denaturation process was found.

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Determination of Glycerol in the Presence of Pentoses

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The use of different carbon chain length compounds as substrates in dehydrogenation and fermentation studies (1) conducted with *Fusaria*, have made it indispensable to possess a dependable method for the determination of glycerol in the presence of pentoses.

At the suggestion of Mr. Nord, it was attempted to adapt the procedure of Fulmer, Hickey, and Underkofler (2) to this purpose, although no data for such determinations were given.

In this paper results are recorded for analyses of glycerol in the presence of *d*-xylose. The method consists of a ceric sulfate oxidation of the pentose and glycerol and a separate determination of *d*-xylose by means of a copper titration method. The amount of glycerol is then calculated by equations or taken directly from graphs.

EXPERIMENTAL

The *d*-xylose was first determined by the method of Shaffer and Somogyi (3), using their reagent of low alkalinity which permits analysis of *d*-xylose in concentrations of 0.01–0.50 mg. (in 5 ml. solution).

The 0.005 *N* sodium thiosulfate was prepared by diluting an approximately 0.1 *N* solution of this reagent standardized against 0.1 *N* potassium iodate solution preserved by the addition of about 10 ml. of 0.1 *N* sodium hydroxide solution per liter.

The samples of *d*-xylose were mixed with the Shaffer-Somogyi reagent and heated in Pyrex test tubes (22 × 250 mm.) in a boiling water bath for 45 minutes instead of the prescribed 35 minutes necessary for glucose. Small beakers were used to cover the test tubes during heating and until titration.

* Communication No. 29.

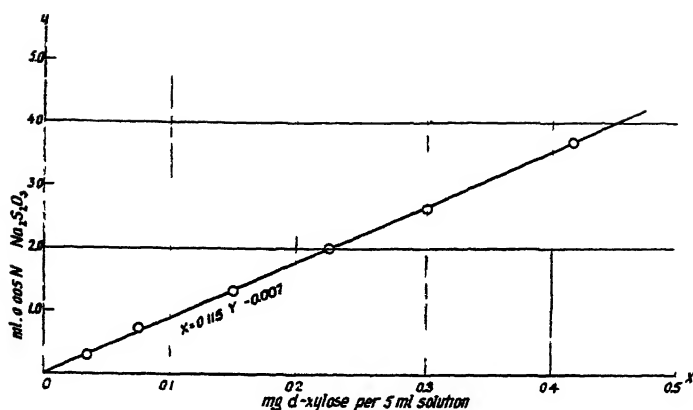
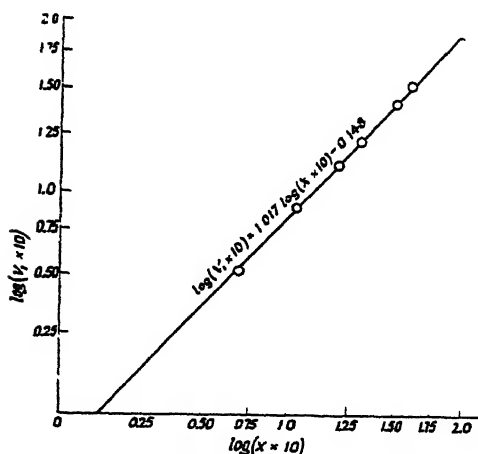


FIG. 1
Determination of Known Amounts of *d*-Xylose

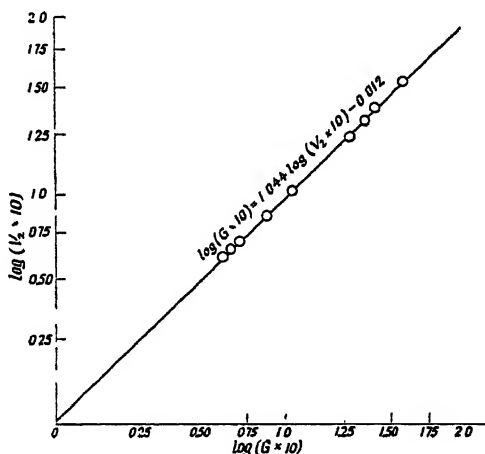


X = mg. *d*-xylose per 5 ml. solution
 V_1 = ml. of ceric sulfate used by the *d*-xylose (mg.)

FIG. 2
Determination of Known Amounts of *d*-Xylose by Ceric Sulfate Oxidation

The *d*-xylose was then oxidized with ceric sulfate in order to permit calculation of the amount used by the glycerol. After measuring 5 ml. of the sugar solution into the test tubes, adding 2 ml. of 1 to 1 sulfuric acid and 5 ml. of 0.1000 *N* ceric sulfate in one molar sulfuric acid and mix-

ing, the tubes were covered and heated in a boiling water bath for 45 minutes. The solutions were then cooled to 20° to 25°C. and the excess



G = mg. glycerol per 5 ml. solution
 V_2 = ml. of ceric sulfate used by the glycerol (mg.)

FIG. 3

Determination of Known Amounts of Glycerol by Ceric Sulfate Oxidation

TABLE I

Determination of Glycerol in the Presence of d-Xylose by Ceric Sulfate Oxidation

mg. <i>d</i> -xylose per 5 ml. soln.	mg. glycerol per 5 ml. soln.	Total ml. ceric sulfate V	ml. ceric sulfate used by <i>d</i> -xylose V_1	ml. ceric sulfate used by glycerol V_2	mg. glycerol found	Error in mg.
3.75	0.22	3.07	2.84	0.23	0.23	+0.01
2.08	1.09	2.60	1.56	1.04	1.12	+0.03
1.67	1.31	2.46	1.24	1.22	1.33	+0.02
1.08	1.61	2.28	0.80	1.48	1.63	+0.02
0.50	1.91	2.03	0.37	1.76	1.94	+0.03

ceric sulfate titrated with ferrous ammonium sulfate, which is kept under an oxygen-free atmosphere. Ferrous *o*-phenanthroline of Smith (4) was used as indicator.

The procedure for the determination of glycerol and mixtures of

d-xylose and glycerol by means of ceric sulfate were identical with that for *d*-xylose.

Best results were obtained by using concentrations in which the total amount of *d*-xylose and glycerol was between 2 and 4 mg. per 5 ml. of solution.

SUMMARY

A method has been devised for the determination of glycerol in the presence of *d*-xylose.

A table and graphs are presented for use in determinations of glycerol, *d*-xylose, and glycerol in the presence of *d*-xylose.

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The Composition of Chyle from a Case of Traumatic Chylothorax

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INTRODUCTION

The thoracic duct transmits chyle from the lacteals of the intestine to the left subclavian vein of the neck, and due to its position relative to vital organs, wounds involving it are usually fatal.

In 1940, Matson and Stacy (1) reported that only about fifty case histories of traumatic chylothorax are on record. These cases generally followed automobile accidents, knife stabs, or gunshot wounds.

Oddly enough there has been little attempt to present a complete chemical analysis of human chyle. Many of the analytical results reported were obtained by the use of older and inadequate methods, and were expressed in terms difficult of interpretation. Most writers consider chyle and lymph to be identical except for lipid content.

One of the earliest chemical analyses of chyle was published in the Archives of Pediatrics for 1907 (2). A reference list of observations on chylothorax and chyle is given in the reference (3) at the end of this paper.

EXPERIMENTAL AND DISCUSSIONS

During December 1939, the rare opportunity to analyze chyle from a case of traumatic chylothorax presented itself.* A white male, 21 years of age, was shot in the chest with a .32 caliber pistol. As a result of the injury to the thoracic duct, chylothorax developed. Between November 23rd and December 24, 18, 290 cc. of fluid was aspirated.

Initially the fluid was bloody and serous, but it gradually became clear and finally milky. It was always sterile on repeated culture.

* The writers are indebted to Drs. Ralph C. Matson and John W. Stacey for supplying the chyle for analysis.

The sample taken for analysis was a slightly coffee colored, creamy fluid and represented the accumulation of several days in the chest

TABLE I

Composition of Human Chyle from a Case of Traumatic Chylothorax

Substance	
Water.....	92 per cent
Solids.....	8 per cent
Protein, total.....	4.73 per cent
Albumin.....	3.72 per cent
Globulin.....	1.01 per cent
A/G ratio.....	3.68
Fibrinogen.....	Trace
Glucose (true sugar).....	35 mg. per cent
Non-protein nitrogen.....	20 mg. per cent
Urea nitrogen.....	12.5 mg. per cent
Uric acid.....	2.3 mg. per cent
Phospholipids.....	197 mg. per cent
Cholesterol, total.....	121 mg. per cent
Cholesterol, free.....	105 mg. per cent
Cholesterol, esters.....	16 mg. per cent
Fatty acids, total.....	2.28 per cent
Fatty acids, free.....	0.73 per cent
Sodium.....	258 mg. per cent
Potassium.....	9.8 mg. per cent
Calcium.....	7.6 mg. per cent
Calcium, diffusible.....	5.9 mg. per cent
Magnesium.....	3.6 mg. per cent
Phosphorus, inorganic.....	10.6 mg. per cent
Chlorides (as Cl ⁻).....	243 mg. per cent
CO ₂ combining capacity at 40 mm.....	60.4 vols. per cent
Phosphatase (acid).....	10.2 Bodansky units
pH (glass electrode).....	7.42
Freezing point.....	-0.60°C.
Specific gravity.....	1.015 at 19°C.
Erythrocytes.....	400,000 per mm ³ .
Leucocytes.....	400 per mm ³ .
Odor.....	none
Color.....	Creamy, slightly coffee colored.

Inoculation on blood agar plates gave no growth.

cavity. The fluid was analyzed for most of the common constituents by generally used and accepted methods. The results are given in Table I.

A consideration of the total acid-base balance of the fluid is of some interest. The freezing point depression of -0.60°C . indicates essential isotonicity with blood. The sodium, potassium, calcium, and magnesium contents respectively represent 122, 2.7, 4.1, and 3.3 milligram equivalents per liter of water, and a total base of 132 milligram equivalents. A total CO_2 content of 60.4 volumes per cent at pH 7.42 and 40 mm. CO_2 tension (assumed in the chest cavity) represents 28 milligram equivalents of HCO_3^- ion. The chloride content was 74 milligram equivalents, and the albumin and globulin present in the fluid at pH

TABLE II

Bases and Acids of Human Blood Serum and Chyle as Milligram Equivalents per Liter of Water*

Bases	Serum	Chyle
Sodium.....	144	122
Potassium.....	5	2.7
Calcium.....	5	4.1
Magnesium.....	1	3.3
Total.....	155	132
Acids		
Chloride.....	111	74
Bicarbonate.....	28	28
Phosphate.....	3	6.3
Protein.....	17	13
Sulfate.....	1	
Total.....	160	121

* Reference (5).

7.42 would bind 13 milligram equivalents of base according to the equations of Van Slyke, Hastings, Hiller, and Sendroy (4). The inorganic phosphorus figured as phosphate buffer at pH 7.42 would bind 6.3 milligram equivalents of base. The total base bound by the above acids is 121 milligram equivalents, leaving $132 - 121 = 11$ milligram equivalents of base to be bound by sulfate and organic acid ions. It is improbable that the sulfate ion bound appreciably more or less base than in blood. However, the high content of free fatty acids in the fluid suggests that appreciable base may have been bound by these. Table II summarizes the above values and gives for comparison the normal values for serum according to Peters (5).

It will be noted that the phosphatase activity of the chyle was high, which may account, in part at least, for the relatively high inorganic phosphate value as compared with blood serum.

The red cell count of the chyle represented approximately eight per-cent contamination with blood.

Since the sample of chyle analyzed had accumulated in the chest over a period of several days it is probable that its composition had been somewhat altered by transfer of material by diffusion into and out of the cavity.

SUMMARY

A sample of human chyle obtained from a case of traumatic chylothorax has been analyzed and the acid-base balance calculated.

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Sulfur in Plants

I. The Effect of Applications of Gypsum and Sodium Selenate on Sulfur Distribution and Manganese, Iron, and Copper Contents of Alfalfa¹

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INTRODUCTION

It is known that sulfur is absorbed by the roots of plants as the sulfate ion which is translocated to the leaf where sulfate-sulfur is reduced. This reduction is believed by Wood (1) to be linked to nitrate-nitrogen reduction and carbohydrate oxidation. Nightengale, Schermerhorn, and Robbins (2), investigating tomato plants grown under sulfur deficient conditions, observed that the sulfur deficient plants possessed a higher content of carbohydrates and nitrates and a lower content of reduced sulfur compounds than the controls. Similar symptoms have been observed by Eaton (3, 4) in the sunflower and in soybeans growing in sulfur deficient nutrient solutions. Since the elements iron, copper, and manganese are involved in plant oxidation-reduction reactions, it was decided to see what effects applications of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ at various levels would have on the uptake of these minerals. In parallel experiments varying amounts of Na_2SeO_4 instead of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ were added to the soil. Painter and Franke (5) conclude that the metabolic behavior of selenium in plants is somewhat similar to that of sulfur. However, there is no evidence for the presence of selenates in plants containing selenium indicating that the manner of reduction of selenate-selenium in plants may be different from that of the reduction of sulfate-sulfur.

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EXPERIMENTAL

Previous experiments conducted on the soils of the Bemidji area by Alway (6) indicate that this region is probably deficient in sulfur. The field chosen for the present work was on a farm near Wilton, Minnesota, in the Bemidji area. This field has had no applications of sulfur fertilizers made to it. On a level plot of one year old alfalfa a section $48' \times 24'$ was selected, and this section was divided into $18 \ 8' \times 8'$ squares. Three squares were reserved as controls and the remainder were subjected to five types of treatment each of which were triplicated. The treatments were made to different $8' \times 8'$ squares as follows: (1) 33 g. C.P. gypsum, (2) 132 g. C.P. gypsum, (3) 3.5 g. Na_2SeO_4 , (4) 7.0 g. Na_2SeO_4 , (5) 17.5 g. Na_2SeO_4 . The gypsum was applied in the powder form while the Na_2SeO_4 was dissolved in water and sprinkled over the plots. The squares for each treatment and control were chosen at random. The alfalfa was cut so that no sample subsequently collected would have sulfur or selenium derived from contamination of the outside of the plant. Applications were on May 4, 1942.

A similar plot was laid out and divided into $8' \times 8'$ squares on the alfalfa field at the University Farm, St. Paul, Minnesota. No gypsum applications were made in this instance. In addition to controls, two treatments of Na_2SeO_4 were made—17.5 g. per square and 35 g. per square.

After three weeks the aerial portions of the plants were cut off at ground level. The samples were air dried, ground to a fine powder in a ball mill and were stored in glass bottles equipped with plastic screw caps.

The following constituents were determined in each sample: moisture, protein nitrogen (Kjeldahl), total sulfur (Parr bomb), total selenium (A.O.A.C.) (7, 8), sulfate-sulfur, reduced sulfur, iron, copper, and manganese.

Total sulfate-sulfur was run as follows: Two grams of sample were treated with 20 ml. of 3 per cent HCl , and the mixture was slowly evaporated to dryness on the steam bath. The water extract of the residue was heated to boiling, and 5 ml. of 5 per cent BaCl_2 were added. After it had stood overnight the precipitate was collected on a Whatman No. 40 filter paper. The paper and precipitate were digested on the steam bath with a mixture of 6 ml. concentrated HNO_3 and 3 ml. 70 per cent HClO_4 . A small flame was used to remove the remaining HNO_3 and most of the HClO_4 . After the residue was cooled and

diluted with water, it was brought to a pH of 3 with dilute NaOH. The mixture was brought to boiling and after standing overnight at room temperature the BaSO_4 was filtered and weighed.

The sulfate-sulfur value was subtracted from the total sulfur value to obtain the reduced sulfur value.

TABLE 1
*Analyses of Alfalfa*¹

Treatment	Ave. wt. per plant ² grams	Protein-N		Total sulfur		Sulfate sulfur		Reduced sulfur	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Wilton Control	0.274	3.98	3.96-4.03	0.25	0.23-0.26	0.04	0.03-0.04	0.21	0.20-0.22
Wilton Gypsum (33 g./8' × 8' square)	0.324	3.98	3.90-4.05	0.31	0.29-0.34	0.09	0.08-0.10	0.22	0.21-0.24
Wilton Gypsum (132 g./8' × 8' square)	0.309	4.16	4.06-4.21	0.38	0.37-0.38	0.13	0.12-0.15	0.25	0.23-0.26
Wilton Selenium (3.5 g. Na_2SeO_4 / 8' × 8' square)	0.439	3.31	3.21-3.47	0.30	0.27-0.34	0.09	0.07-0.12	0.21	0.20-0.22
Wilton Selenium (7.0 g. Na_2SeO_4 / 8' × 8' square)	0.325	3.58	3.48-3.65	0.45	0.44-0.47	0.21	0.19-0.23	0.24	0.22-0.26
Wilton Selenium (17.0 g. Na_2SeO_4 / 8' × 8' square)	0.323	3.62	3.39-3.76	0.52	0.44-0.58	0.25	0.20-0.29	0.27	0.24-0.28
University Farm Control ³	0.141	4.08		0.50		0.22		0.28	
University Farm (17 g. ³ Na_2SeO_4 / 8' × 8' square)	0.188	4.28		0.57		0.53		0.34	
University Farm (35 g. ³ Na_2SeO_4 /8' × 8' square)	0.179	4.47		1.04		0.62		0.42	

¹ Ranges given in the above table represent the extremes of the triplicated squares. Maximum variation between duplicate analyses did not exceed 2 per cent.

² Obtained by dividing the total sample weight by the number of plants in the sample.

³ The University Farm samples from the triplicated plots were combined together to form a single composite sample.

Colorimetric methods employing the Coleman Universal Spectrophotometer were used in the determination of the minerals. The α, α' -dipyridyl method modified by Andrews and Felt (9) was used for the determination of iron. The readings of per cent transmittance were made at a wave length of 517 m μ .

Previous to the copper and manganese determinations, the samples were wet ashed as follows: Six grams of the dried plant material were placed in a 300 ml. Kjeldahl flask, and 25 ml. 70 per cent analytical HNO_3 were added. When reaction had subsided, 6.5 ml. 98 per cent

analytical H_2SO_4 were added and after rotating to insure mixing, the mixture was gently heated with a low flame. The flame was removed when the mixture began to char and after the solution had cooled, 10 ml. HNO_3 and 4.5 ml. 70 per cent analytical HClO_4 were added. Gentle heating was resumed, and soon brown fumes of NO_2 appeared. After

TABLE II
Analyses of Alfalfa¹

Treatment	Selenium		Copper		Iron		Manganese		Ratio mol N mol	Ratio mol Total S	Ratio mol N mol Red.- S X Mn
	Mean $\mu\text{g. g.}$	Range $\mu\text{g. g.}$	Mean $\mu\text{g. g.}$	Range $\mu\text{g. g.}$	Mean $\mu\text{g. g.}$	Range $\mu\text{g. g.}$	Mean $\mu\text{g. g.}$	Range $\mu\text{g. g.}$	Red.-S	Red.-S	
Wilton Control			7.9	7.7-8.1	209	203-217	85	84-87	43	1.2	275
Wilton Gypsum (33 g. $\text{S}' \times \text{S}'$ square)			7.6	6.7-8.1	215	212-230	95	91-97	41	1.4	238
Wilton Gypsum (132 g. $\text{S}' \times \text{S}'$ square)			7.5	7.4-7.7	214	200-234	91	83-98	39	1.6	235
Wilson Selenium (3.5 g. Na_2SeO_4 $\text{S}' \times \text{S}'$ square)	92	82-106	6.0	5.9-6.0	189	181-194	77	76-79	36	1.4	257
Wilton Selenium (7.0 g. Na_2SeO_4 $\text{S}' \times \text{S}'$ square)	190	170-212	5.4	4.9-5.5	183	179-185	74	72-76	35	1.5	255
Wilton Selenium (17 g. Na_2SeO_4 $\text{S}' \times \text{S}'$ square)	309	226-347	5.3	5.2-5.4	189	185-192	71	70-73	31	1.9	237
University Farm Control	—		9.5		337		69		33	1.8	266
University Farm (17 g. Na_2SeO_4 $\text{S}' \times \text{S}'$ square)	234		—		226		62		29	2.6	255
University Farm (35 g. Na_2SeO_4 $\text{S}' \times \text{S}'$ square) ...	314		—		326		56		24	2.5	239

¹ Ranges given in the above table represent the extremes of the triplicated squares. Maximum variation between duplicate analyses did not exceed 2 per cent.

a few minutes the fuming ceased, and the solution began to char. The flame was made as low as possible, and heating was continued until all traces of free carbon had disappeared and the solution had turned to a bright yellow color. At this point stronger heating was applied until the excess HClO_4 had been removed. Thirty ml. of water were added to the cooled solution. The heat generated by the dilution was allowed

to subside, and the mixture was transferred to a 100 ml. volumetric flask and brought to volume. Insoluble silica was removed by centrifuging at 1500 R.P.M. for 20 minutes. Aliquot parts of this solution were used for the determination of copper and manganese.

Copper was determined by the carbamate method as modified by Eden and Green (10) and Greenleaf (11). The per cent transmittance through copper carbamate-amyl alcohol solution was determined at a wave length of 458 $m\mu$.

The method of Richards (12) for manganese was modified. Forty ml. of the ashing solution were transferred to a pyrex 50 ml. volumetric flask containing 0.5 g. KIO_4 . Oxidation of the manganese salts to permanganic acid was facilitated by heating the flask on the steam bath for 4 hours. The cooled flasks were made up to volume and the per cent transmittance through the permanganic acid was determined at a wave length of 533 $m\mu$.

Complete blanks containing the reagents used were employed for each method. Evaluation of the unknown was accomplished by comparison with standard per cent transmittance-concentration curves which were prepared for each method. All determinations were made in duplicate.

DISCUSSION

Alway (6) demonstrated that severe chlorosis and retardation of growth of alfalfa in some regions of the Bemidji area could be alleviated by application of various sulfates or elemental sulfur to the soil. Total sulfur values of the treated alfalfa showed an increase over the untreated but this fact alone does not conclusively prove that sulfur is the limiting element. Wood (1) has shown that sulfates accumulate in plants without entering into metabolic activity. Deficiencies of other elements such as iron (13) cause the plant to exhibit symptoms similar to that of sulfur deficiency.

In the present work the application of gypsum to the Wilton plots produced a decided stimulation of growth as well as in total sulfur content. The greatest amount of total sulfur was found in the case of the heavier application. The untreated alfalfa was apparently normal, the usual sulfur deficiency symptoms of chlorosis and spindly growth being absent.

The increase in total plant sulfur upon gypsum treatment can be accounted for to a large extent as sulfate. There was a small increase in reduced sulfur, especially in the heavier application. The manganese

content of treated plants was somewhat greater than that of the untreated but this did not follow, in extent, the increase of reduced sulfur. The amounts of manganese found are unusually high, and the values for iron are low when compared with alfalfa analyses reported in the literature.

The addition of Na_2SeO_4 to the Wilton plots produced an acceleration of growth which was most evident in the lowest selenate application. This fact is in agreement with the findings of Trelease and Trelease (14) who have demonstrated that additions of inorganic selenium to the nutrient solutions will stimulate the growth of *Astragalus racemosus* and *Astragalus pattersonii*. These investigators have also presented evidence which seems to indicate that the growth response is most pronounced when an optimum sulfur-selenium ratio is reached in the culture media.

Total sulfur uptake was increased by the selenate treatment and there was a greater amount of reduced sulfur in this case than in the gypsum treatments. Upon examining the total sulfur/reduced sulfur ratios it will be noted that the reduced sulfur is a smaller fraction of the total sulfur in the selenized plants than in the gypsum treated plants. It is difficult to consider this particular field sulfur deficient in view of the above results. Manganese uptake was diminished in the plants receiving selenate treatment in contrast to the increase noted in the case of gypsum treatment. The iron content, however, was similarly lowered as was copper. It had been noted earlier that applications of selenate to chlorotic and stunted alfalfa in the Bemidji area brought about the return of full green color and increased its growth. The nitrogen/reduced sulfur ratios brought about by gypsum and selenate treatment indicate that protein in the plant is becoming richer in sulfur or that non-protein sulfur in some reduced form has increased.

Applications of Na_2SeO_4 to the University Farm plots caused a great increase in the total sulfur, sulfate-sulfur, and reduced sulfur of alfalfa. The proportion resulting as sulfate-sulfur was greater than in any of the Wilton plants. Growth stimulation and reduction of manganese content resulted here as well as in the Wilton plants. The iron content was somewhat higher here.

While the Wilton and University Farm alfalfa are not strictly comparable, certain general comparisons are of interest. The weights of individual plants were lower at the University Farm but this may be due to a heavier stand. The values for nitrogen/reduced sulfur \times manganese appear to be fairly constant for all plots regardless of treat-

ment. This would seem to indicate that the amount of nitrogen and reduced sulfur is related to the manganese content. Manganese is known to be toxic in high amounts. It is possible that reduction of manganese uptake by application of selenate is due to pH changes in the soil making manganese less available. It is possible also that additions of the sodium ion may exert a "mutual replacement" of the manganese ion. This hypothesis is in accordance with the views of Collander (15).

SUMMARY

Applications of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and Na_2SeO_4 to the soil were able to increase total sulfur uptake by alfalfa, but the amount of reduced sulfur did not increase as readily. Other factors play a role in the sulfur metabolism, and it appears likely that manganese influences the extent of such metabolism. Stimulating effects of Na_2SeO_4 may very well be due to a lowering of manganese content. Abnormally high manganese content was accompanied by low iron values.

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Growth Factor Requirements of Clostridia*

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INTRODUCTION

The nutrition of the acetone-butyl alcohol producing organism *Clostridium acetobutylicum* has been studied in detail, largely because of its industrial significance. Work in this laboratory (1) demonstrated that this organism on a synthetic basal medium of glucose, asparagine, and mineral salts required biotin and an unidentified factor from yeast (which was termed BY) for maximum growth. Rubbo and Gillespie (2) isolated a factor from yeast essential for this organism. This they showed to be *p*-aminobenzoic acid (P.A.B.) and stated that it was the only growth factor required for the nine strains they tested. Lampen and Peterson (3) reported that P.A.B. is probably identical with the BY factor, but that biotin as well is essential for growth. Park and Wood (4) confirmed the need for both growth factors.

In this paper we wish to report the growth requirements of a number of clostridia. The growth-promoting power of a series of compounds related to P.A.B. has also been tested.

EXPERIMENTAL

Handling of Cultures: The stock cultures were kept on sterile soil, and a loopful was inoculated into 10 cc. tubes containing the basal medium plus 0.5 per cent peptone. At 24 hrs. these tubes were centrifuged and the cells resuspended in 10 cc. of freshly sterilized 0.9 per cent saline. Two drops of this washed culture were added to each tube in the testing of the various species. For the determina-

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tion of the activities of the homologs, the inoculum was reduced to one drop per tube.

Basal Medium: This contained the following amounts per 100 cc.: 2.0 g. of glucose, 0.1 g. $(\text{NH}_4)_2\text{HPO}_4$, 0.2 g. ammonium acetate, 0.1 g. mineral salts (K_2HPO_4 10 g., KH_2PO_4 10 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 4 g., NaCl 0.2 g., $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.2 g., $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g.) and 5.0 mg. $\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$. The medium was adjusted to $\text{pH } 6.7 \pm 0.1$ before autoclaving. 19×150 mm. tubes were used in which about 5 mg. of reduced iron had been placed. The cultures were incubated anaerobically in oat jars at 37° .

Purified hydrolyzed casein was prepared by stirring 100 cc. of a 10 per cent solution of HCl-hydrolyzed Labco "vitamin-free" casein with 2 g. of norite for 30 min. at 100° , then cooling and filtering. This filtrate was brought to $\text{pH } 2$ with H_2SO_4 and continuously extracted with ether for 36 hrs. The residue was brought back to 10 per cent concentration and stored in the refrigerator. This material is free of P.A.B.

Growth Requirements of Clostridium Species: Most organisms of the butylicum type are able to attain maximum growth with the addition of biotin alone (Table I). *Cl. butylicum* No. 28 however, is unable to grow unless both P.A.B. and biotin are present. *Cl. felsineum* (Carbone) No. 41 grew slowly with biotin as the only addition, but never reached the maximum turbidity attained in the presence of biotin and P.A.B. Slight growth occurred with several strains on the unsupplemented basal. Strain No. 40, tested because it is an excellent pectin-fermenter, requires only biotin.

Cl. sporogenes A.T.C. No. 459 required only biotin but three other sporogenes strains and *Cl. tertium* would not grow even when both P.A.B. and biotin were present. A situation similar to the response of the sporogenes strains was observed by McDaniel (5) with various strains of *Cl. tetani*. A.T.C. No. 457 grew well with only biotin added but three other strains gave little or no response to biotin additions alone. When the other growth-factor requirements of these strains are known it is probable that biotin also will appear in the required list.

All strains of *Cl. acetobutylicum* required the addition of both P.A.B. and biotin for the attainment of any more than mere traces of growth, or for growth in subculture. The American Type Culture No. 862 strain is one of those used by Rubbo and Gillespie (2) who reported growth with P.A.B. alone. We are unable to offer any explanation for this discrepancy in results.

Activity of Compounds Related to P.A.B.: A microbiological assay for P.A.B. employing *Cl. acetobutylicum* S9 as the test organism has been developed in this laboratory (6) and it was desirable to test these

homologs under conditions used for the assay of natural materials. For these tests 0.01 γ of biotin, 5 mg. of the purified hydrolyzed casein, 1 mg. of cystine, and 750 γ of tryptophan were added per 100 cc. of medium.

TABLE I
Growth Requirements of Various *Clostridia*

Microorganism**	None	Additions to basal medium*		
		Biotin (0.001 γ /cc.)	P.A.B. (0.05 γ /cc.)	Biotin + P.A.B.
<i>Bacillus butylicus</i> No. 39 (Fitz)	87	32	84	28
<i>Bacillus saccharobutyricus</i> von Klecki No. 75 . . .	87	35	81	30
<i>Clostridium acetobutylicum</i> S9	99	97	98	40
“ “ , A.T.C. No. 862	99	98	94	30
“ “ , “ “ “ (†)	96	93	95	49
“ “ , “ “ “ 824	99	96	98	48
“ “ , “ “ “ (†)	97	94	98	40
“ “ , “ “ “ 3625	94	92	88	43
“ “ BF	98	91	96	48
“ “ L	94	96	93	34
“ “ Pike	96	94	95	43
<i>Clostridium butylicum</i> No. 16	85	16	85	17
“ “ “ 21	97	20	96	20
“ “ “ 22	81	30	80	29
“ “ “ 28	96	97	92	34
“ “ “ 37 (Fernbach)	92	34	89	30
“ “ “ 64	82	29	84	21
“ “ “ 69	91	18	89	20
<i>Clostridium felsineum</i> No. 41 (Carbone)	80	67	83	35
<i>Clostridium</i> sp. No. 40	91	21	90	24
<i>Clostridium sporogenes</i> , A.T.C. No. 459	90	23	—	—
<i>Clostridium tetani</i> A.T.C. No. 457	90	23	—	—

* Turbidity measurements were made with an Evelyn photoelectric colorimeter using a 660 $m\mu$ filter. Growth values are expressed as percentage transmission against the uninoculated basal set at 100. Read after 72 hours incubation.

** Those cultures marked † were obtained from Dr. J. L. Stokes of Merck and Company. All other cultures were furnished by Dr. Elizabeth McCoy of the Department of Agricultural Bacteriology.

The compounds were added to the medium before autoclaving. The data are given in Table II.

Rubbo, *et al.* (7) report the ethyl ester of P.A.B. to be of equal activity with the free acid. Both the ethyl and methyl esters show less than 0.1 per cent activity with our organism. The more complex ester procaine was 10–20 per cent as active on a molar basis. *N*-Acyl deriva-

TABLE II
Activity for *Cl. acetobutylicum* S9 of Compounds Related to P.A.B.

Compound	Formula	Level tested mγ/cc.	% P.A.B. activity	
			per gram	per mole
Methyl <i>p</i> -aminobenzoate	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{COOCH}_3$	50	<0.1	<0.1
Ethyl <i>p</i> -aminobenzoate	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{COOC}_2\text{H}_5$	20	<0.1	<0.1
Procaine	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{COOCH}_2\text{CH}_2-\text{N}(\text{C}_2\text{H}_5)_2, \text{HCl}$	2.0	5-10	10-20
<i>N</i> -Benzoyl <i>p</i> -aminobenzoic acid	$\text{C}_6\text{H}_5-\text{CONH}-\text{C}_6\text{H}_4-\text{COOH}$	20 0.10*	<0.6 55	<1.0 98
<i>N</i> -Acetyl <i>p</i> -aminobenzoic acid	$\text{CH}_3\text{CONH}-\text{C}_6\text{H}_4-\text{COOH}$	250† 250 0.10*	0.03 0.05 63	0.04 0.08 90
<i>p</i> -Nitrobenzoic acid	$\text{O}_2\text{N}-\text{C}_6\text{H}_4-\text{COOH}$	0.15	70-80	90-100
P.A.B.- <i>N</i> - <i>d</i> -riboside	$\text{HOOC}-\text{C}_6\text{H}_4-\text{NH}-\text{C}_5\text{H}_9\text{O}_4$	0.2† 0.2	44 49	87 96
P.A.B.- <i>N</i> - <i>l</i> -arabinside	$\text{HOOC}-\text{C}_6\text{H}_4-\text{NH}-\text{C}_5\text{H}_9\text{O}_4$	0.2	53	104
Pantocaine	$\text{C}_6\text{H}_5-\text{NH}-\text{C}_6\text{H}_4-\text{COOCH}_2\text{CH}_2-\text{N}(\text{CH}_3)_2, \text{HCl}$	25 1.0*	0.4 14-16	0.8 26-30
<i>p</i> -Aminophenylacetic acid	$\text{H}_2\text{N}-\text{C}_6\text{H}_4-\text{CH}_2\text{COOH}$	5,000	0.002	0.002

* Hydrolyzed by autoclaving with 2*N* NaOH for 2 hrs.

† Sterilized by filtration.

tives showed only slight activity. On hydrolysis these gave 90-100 per cent of the theoretical P.A.B. activity per mole. Sterile filtration

of *N*-acetyl P.A.B. and addition to the sterilized basal caused a 50 per cent drop in activity from that of the material sterilized by autoclaving, indicating a slight hydrolysis during autoclaving.

Rubbo, *et al.* (7) report only 10 per cent activity for the *p*-nitro derivative. Under our conditions the compound has a potency equivalent to P.A.B. on a molar basis, although duplicate tubes show a wider variation. The *N*-glycosides are apparently readily hydrolyzed by the organism and show molar activity equivalent to that of P.A.B. Pantocaine was the only *N*-alkyl derivative tested. The intact compound shows little activity, but after removal of the dimethylethanolamine portion by hydrolysis the residue was about one-fourth as potent as P.A.B.

p-Aminophenylacetic acid has been reported to be ten times as active as P.A.B. (7). The compound as commercially obtainable (Eastman Kodak Company) had approximately 0.1 per cent of the potency of P.A.B. After two recrystallizations this value dropped to 0.002 per cent. This approximate ratio has also been found with *Cl. acetobutylicum* strains D, BF, L, A.T.C. No. 824, and A.T.C. No. 3625. When tested on *Acetobacter suboxydans*, which also requires P.A.B. (8), *p*-aminophenylacetic acid showed 2 per cent of the potency of P.A.B.

Wyss, *et al.* (9) have recently reported *p*-aminophenylacetic acid to possess 0.1 per cent of the potency of P.A.B. for *Cl. acetobutylicum* A.T.C. No. 3625. Of a number of substituted *p*-aminobenzoic acids tested they found the 2-F derivative to be one-third as active as P.A.B. with the 2-Br, 2-I, and 3-COOH compounds exhibiting traces of activity.

The following compounds were tested and proved inactive at the concentrations per cc. stated: *o*-aminobenzoic acid (3γ), isonicotinic acid (10γ), *p*-hydroxybenzoic acid (2γ), urethane (600γ), *N*-phenylurethane (3 mg.), guanine (5γ), adenine (5γ), xanthine (10γ), uracil (10γ), uric acid (10γ), adenylic acid (10γ), *d*-ribose (20γ), *dl*-arabinose (10γ), *d*-xylose (10γ), glucuronic acid (10γ), vitamin C (10γ), and a folic acid concentrate (1.6 mγ). A mixture of β-alanine, calcium pantothenate, riboflavin, pyridoxin, thiamin, inositol, pimelic acid, glutathione, and traumatic acid likewise did not support growth. Sulfanilamide at 1.0 mγ per cc. was inactive, and 80–100 per cent recovery of added P.A.B. was obtained in the presence of this level of sulfanilamide. Thus the stimulatory action of low concentrations of sulfanilamide reported by Green and Bielschowsky (10) for *Br. abortus* could not be demonstrated with *Cl. acetobutylicum*.

Synthesis of B-vitamins by Cl. acetobutylicum S9: The synthesis of other B vitamins by the S9 strain has been measured on the basal medium

plus 0.002 γ biotin and 0.005 γ P.A.B. per cc. A 10 cc. tube of this synthetic medium was inoculated from a peptone culture and at 18 hours a 1 per cent inoculum of the synthetic transfer was made into the test flasks. After 48 hours incubation the flasks were steamed and the entire fermented medium analyzed. The vitamin contents found were: pyridoxin 36 m γ . per cc., pantothenic acid 0.16 γ per cc., folic acid 10 m γ . of 40,000 unit potency material per cc., riboflavin 0.18 γ per cc., thiamin 0.015 γ per cc., and nicotinic acid 0.6 γ per cc. Rodgers, using a corn mash medium, was able to obtain 20 γ of riboflavin per cc. with this organism (11). While the amounts produced under these conditions are small, the organism definitely synthesizes the other B vitamins which it does not require preformed.

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SUMMARY

1. The growth factor requirements of twenty strains of *Clostridia* have been tested. Biotin is essential for all of these organisms. In addition seven strains of *Cl. acetobutylicum*, *Cl. butylicum* No. 28, and *Cl. felsineum* (Carbone) No. 41 need P.A.B. for maximum growth.

2. The ability of a series of compounds related to P.A.B. and of a number of other nutritionally important compounds to replace P.A.B. in the growth of *Cl. acetobutylicum* S9 has been determined.

3. On the synthetic basal medium employed the S9 strain synthesizes those B vitamins which it does not require preformed for growth.

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Experiments on the Application of *Neurospora sitophila* to the Assay of Pyridoxin in Tomato Plants

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INTRODUCTION

The induction, by means of x-rays, of a mutant of *Neurospora sitophila* for which pyridoxin is an essential growth supplement has been reported by Beadle and Tatum (1941). The present paper concerns attempts to use this mutant organism in the assay of the pyridoxin contents of tomato plants.

METHODS

The stock culture of *Neurospora sitophila* of the "pyridoxinless" strain¹ was maintained on basal medium (see below) containing pyridoxin and solidified with agar. Inoculation of the experimental cultures was accomplished by removal of a wire loop full of spores (from a stock culture 4 to 14 days old) to sterile water and the pipetting of 0.1 cc. of the spore suspension into each culture. Density of the spore suspension between wide limits did not appear to influence subsequent growth of the culture.

The basal medium used was that suggested by Beadle and Tatum (1941) and contained per liter of redistilled water: 2 g. KH_2PO_4 , 10 g. ammonium tartrate, 2 g. NH_4NO_3 , 1 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g. NaCl , 0.26 g. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg. FeCl_3 , and 30 g. sucrose. In addition 4 micrograms of crystalline biotin methyl ester were supplied per liter since this material is essential to the growth of *Neurospora*. The basal medium, which was prepared in 1 or 2 liter lots and preserved under toluene, is of twice the required final concentration and was diluted before use as described below. The initial pH of the basal medium was 5.3.

All cultures were made in liquid medium contained in 50 cc. Erlenmeyer flasks. Five cc. of the above basal medium was dispensed into each of a series of such flasks. Pyridoxin or other supplement was then added from a stock solution and the total volume in each flask made up to 10 cc. The flasks were plugged with

¹ The original cultures of this organism were supplied by Professors G. W. Beadle and E. L. Tatum, Stanford University.

cotton, autoclaved at 15 pounds pressure for 15 minutes, inoculated as described above, and incubated. At the end of the incubation period the mycelial mat was removed from each flask, washed thoroughly with distilled water, dried rapidly at 80°C. and weighed to 0.2 mg.

Pyridoxin not sterilized by heat and added after sterilization of the basal medium was as effective in promoting growth as was pyridoxin sterilized in the basal medium, indicating that the observations made by Snell (1942) on *Streptococcus lactis* R do not hold true for *Neurospora*.

Temperature. The first 37 experiments were incubated at a temperature of 25–26°C. After trials at 36, 32, and 29°C., the latter temperature was selected as giving more rapid growth under the conditions used than either 32° or 25–26°. The last 22 experiments were incubated at either 29°C. or at 30°C.

TABLE I

Reproducibility of Replicate Cultures of Neurospora sitophila Grown on Medium Containing Pyridoxin

Each figure represents one determination. Incubated 2.0 days at 29°C.

Expt. No.	γ B ₆ /10 cc. Replicate	0.00	0.05	0.10	0.15	0.20	0.30
			mg. dry weight of mycelial mat per 10 cc.				
54	1	0.4	3.0	6.7	10.0	11.0	14.3
	2	0.3	3.4	6.9	9.4	—	13.0
	3	0.3	3.6	7.3	10.2	11.8	13.9
	4	0.4	3.8	7.1	10.9	11.1	15.5
	Av.	0.35	3.4	7.0	10.1	11.3	14.2
55	1	0.6	4.8	7.7	10.1	11.8	12.0
	2	0.6	4.0	7.1	10.3	12.0	13.4
	3	0.7	4.1	6.8	11.6	11.0	14.0
	4	0.5	4.1	7.8	11.4	11.8	14.4
	Av.	0.6	4.2	7.4	10.8	11.6	13.5

Reproducibility. Table I gives data from two experiments showing the agreement between mycelial dry weights from replicate flasks at different levels of pyridoxin concentration. The individual determinations vary, in the mean, by approximately 6% from the group means of 4 determinations. The response to pyridoxin obtained in different experiments varied somewhat, as is apparent from the following tables. Control series similar to those of Table I were included in each experiment.

Growth for Varying Periods. Table II gives data on the growth of *Neurospora sitophila* ("pyridoxinless"), at a temperature of 25–26°C., with varying additions of pyridoxin and over varying periods of time. At this temperature, mycelial dry weights increased rapidly during the

first 3 days, and then increased more slowly up to 7 days. Further slow increases persisted after 7 days. Over a period of weeks, mycelia weighing several mg. were obtained even from cultures containing no added pyridoxin.

TABLE II

Growth of Neurospora sitophila at 25-26°C. in Medium Containing Various Amounts of Pyridoxin and Cultured for Various Periods

Each figure represents 4 to 16 determinations

Microgram B ₆ /10 cc.	Days 3	4	5	6	7
		<i>mg. dry weight of mycelium per 10 cc.</i>			
0.00	0.3	0.1	0.3	0.9	0.8
0.05	4.4	4.3	4.5	5.5	5.7
0.10	6.6	7.5	8.6	9.0	9.7
0.20	11.9	14.8	16.5	15.0	17.5
0.30	16.6	20.1	23.2	26.7	—

TABLE III

Growth of Neurospora sitophila on Medium Containing Pyridoxin or Containing Tomato Leaf Extracts

Each 3 day figure based on 4 determinations; each 5 day figure based on 10 determinations. Temperature 26°C.

Pyridoxin series			Tomato leaf extract series				
γ B ₆ /10 cc.	Wt. of mycelium		Tomato leaf represented in sample	Wt. of mycelium		Apparent B ₆ per sample	
	3 days	5 days		3 days	5 days	3 days	5 days
	<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	<i>micro-gram</i>	<i>micro-gram</i>
0.00	0.0	0.0	0.0	0.0	0.0	0.000	0.000
0.05	3.2	4.1	5.0	4.0	4.6	0.004	0.062
0.10	6.0	6.8	10.0	6.0	7.0	0.097	0.098
0.20	12.1	12.9	20.0	9.2	11.8	0.152	0.158
0.30	16.1	17.8					

Response to Tomato Leaf Extract. The tomato leaves analyzed in this and subsequent experiments were obtained from vigorous greenhouse grown plants. The leaves were dried rapidly in a current of air at 60°C., and were then ground in a Wiley mill. Extraction of the sample was accomplished by autoclaving in distilled water (4 mg. sample per cc.) at a pressure of 15 lbs. for 15 minutes. Table III shows the growth of *Neurospora* on basal medium supplemented with extracts representing

3 different amounts of dried leaf sample. It is evident that the leaf extract contains pyridoxin or a material capable of substituting for pyridoxin in the nutrition of the *Neurospora*. However, the apparent pyridoxin concentration in tomato leaf extract decreased as the sample size increased. Table IV gives the recovery of added pure pyridoxin in the presence of tomato leaf extract. In the experiments harvested 3 days (at 25–26°C.) after inoculation, this recovery was independent of the amount of added pyridoxin and approximated 100%. In the ex-

TABLE IV

Per cent Recovery of Various Amounts of Added Pyridoxin in the Presence of Various Amounts of Tomato Leaf Extract and at Varying Times

Temperature 26°C. Each 3 day figure based on 4 determinations, each 5 day figure on 10 determinations

Tomato leaf represented in extract per flask mg.	B ₆ added per flask γ	Recovery of added vitamin	
		after 3 days per cent	after 5 days per cent
5	0.06	97	132
	0.10	96	140
	0.15	98	130
		<hr/> 97	<hr/> 134
10	0.06	97	168
	0.10	106	179
	0.15	117	165
		<hr/> 107	<hr/> 171
20	0.06	110	167
	0.10	111	180
	0.15	96	—
		<hr/> 106	<hr/> 174

periments harvested 5 days after inoculation, recovery was also independent of the amount of added pyridoxin but varied between 130 and 180%. Results similar to those obtained with 3 day incubation at 25–26°C. were obtained with 2 day incubation at 29–30°C. Although the data of Table III suggest that tomato leaf extract may contain substances toxic to *Neurospora* still such a hypothesis is not born out by the data of Table IV which suggests in fact that tomato leaf extract, when tested in 5 day experiments may contain substances which increase the response of *Neurospora* to pyridoxin. It will now be shown that it

is possible that tomato leaf extracts may contain both toxic substances and growth stimulatory substances other than pyridoxin.

Effect of Casein Hydrolyzate. Table V shows that the addition of casein hydrolyzate (0.4%) increases the growth of *Neurospora* on medium containing pure pyridoxin. This effect of casein hydrolyzate could not be duplicated by additions of asparagine, riboflavin, pantothenic acid, or *p*-amino benzoic acid. Experiments done after the completion of the other work reported in this paper have however shown, in confirmation of the work of Stokes, Foster, and Woodward (1943) that the effect of casein hydrolyzate can be largely simulated by the

TABLE V

Effect of Casein Hydrolyzate (0.4%) on the Growth of Neurospora sitophila in Medium Containing Pyridoxin and/or Tomato Leaf Extract

Each figure based on 4 determinations. Incubated 2.0 days at 30°C.

Effect of casein hydrolyzate on response to pyridoxin			Response to tomato leaf extract in medium containing casein hydrolyzate				
B ₆	Mycelium		Leaf ex.	B ₆	Mycelium	Apparent B ₆	Recovery
	- casein	+ casein					
$\gamma/10$ cc.	mg./10 cc.	mg./10 cc.	mg./10 cc.	$\gamma/10$ cc.	mg./10 cc.	$\gamma/10$ cc.	per cent
0.00	0.00	0.00	5	0.00	4.60	0.050	
0.05	3.15	4.60	5	0.10	7.85	0.091	41
0.10	5.75	8.55	10	0.00	6.70	0.077	
0.15	7.70	10.85	10	0.10	9.20	0.112	35
0.20	9.70	13.05	20	0.00	9.15	0.111	
0.30	11.35	14.65	20	0.10	10.80	0.140	29

addition of thiamin to the basal medium. Casein hydrolyzate at a concentration of 0.4% gave a larger effect than lower concentrations, while still higher concentrations increased the controls (no added pyridoxin) markedly. In Table V it may also be seen that in contrast to the results of Table IV, the recovery of added pyridoxin in the presence of leaf extract and casein hydrolyzate was only 41-29%. The data of Tables IV and V taken together suggest that the basal medium is deficient in some substance other than pyridoxin but contained in casein hydrolyzate or tomato leaf extracts. That the recovery of added pyridoxin approximated 100% in the 3 day cultures of Table IV may have been owing to a fortuitous balance between growth inhibitory

substances and growth stimulatory substances other than pyridoxin contained in the tomato leaf extract.

Toxicity of Extract. Some effort was expended in attempting to discover a simple way of obtaining tomato leaf extracts containing pyridoxin but free of materials causing low recovery of added pyridoxin. To this end samples of leaf were extracted with acetone or with 100 per cent, 95 per cent, and 80 per cent alcohol. These extracts appeared to contain most of the pyridoxin and also the bulk of the interfering sub-

TABLE VI

Effect of Treatment With Neutral Lead Acetate on the Apparent Pyridoxin Content and Recovery of Pyridoxin in Tomato Leaf Extracts

Assay in medium containing casein hydrolyzate. Each value based on 8 determinations

Sample represented in extract	Apparent micrograms pyridoxin in sample ¹		Per cent recovery of 0.1 microgram added pyridoxin		Micrograms per g. sample, corr. for recovery	
	Plain extract	Pb acetate treated ext.	Plain extract	Pb acetate treated ext.	Plain extract	Pb acetate treated ext.
mg.	microgram	microgram	per cent	per cent	microgram/g.	microgram/g.
5	0.050	0.083	66	94	15.2	17.7
10	0.086	0.160	54	97	15.9	16.5
20	0.133	0.259	39	89	17.0	14.6
Average.....					16.0	16.3

¹ These values corrected for a 14.5 per cent destruction of pyridoxin in the lead acetate treatment.

stances. Charcoal adsorption of the water extract removed the interfering substances, as well as the pyridoxin. Neutral lead acetate treatment of the extract however appeared to selectively remove toxic interfering substances from the extract. Aqueous extracts were treated with an excess of neutral lead acetate, filtered, and the excess lead removed with H₂S. Table VI gives a comparison of apparent pyridoxin contents and recoveries of added pyridoxin for extracts treated and not treated with lead acetate. The non lead acetate treated extract gave low recoveries of added pyridoxin. The recovery decreased with increasing sample size. Lead acetate treated extracts yielded higher

values for apparent pyridoxin and recoveries of 89–97%. The calculated pyridoxin content of the original ground leaf material appears to be the same for the two methods of assay.

The data of Table VI support the view that tomato leaf extract contains materials inhibitory to the growth of *Neurospora* which complicate the use of this organism in the assay of pyridoxin. The extent of the

TABLE VII

Extraction of Pyridoxin from Dried Tomato Leaves by Various Methods

Each figure based on 2 determinations

- A. Dry leaf samples placed directly in nutrient medium in culture flask.
 B. Dry leaf samples extracted by autoclaving in water for 15 minutes at 15 lbs. and water extract placed in culture flask.
 C. Successive 15 minute extractions of the same 10 mg. sample of dried tomato leaf.

Sample per flask	A. Dry sample in culture flask	B. Extract of sample in culture flask	Per cent extraction by B relative to A	C. Successive extraction of same sample		
				Number of extraction		Per cent of total extracted
mg.	$\gamma B_6/\text{sample}^1$	$\gamma B_6/\text{sample}^1$			$\gamma B_6/\text{sample}^1$	
5	0.068	0.054	79	1	0.094	89
10	0.120	0.094	78	2	0.011	10
20	0.188	0.165	88	3	0.001	1
				4	0.000	0
Total.....					0.106	

¹ Not corrected for recovery.

inhibition can apparently be adequately estimated from recovery experiments.

Completeness of Extraction. Table VII shows that when samples of dried tomato leaf are weighed directly into the basal medium so that extraction is performed during autoclaving of the culture flasks, and possibly by the growing *Neurospora*, the apparent pyridoxin content of the tissue is larger than when water extracts prepared by autoclaving (4 mg. sample per cc. of H₂O) are assayed. The results indicate that at least 10–20% of the pyridoxin is not extracted by one 15 minute autoclaving at 15 pounds pressure. Table VII also shows that if the

same sample is extracted for repeated 15 minute intervals, 89% of the total extractable pyridoxin is obtained in the first extraction and the bulk of the remainder in the second extraction. In the making of extracts the ratio of leaf tissue to water between the limits 2 to 6 mg. per cc. was found to be immaterial to completeness of extraction.

Application of Assay. Consideration of the results of Table VI led to the conclusion that large scale analysis of plant extracts for pyridoxin might be practicable without removal of interfering materials with lead acetate, provided that recovery experiments were made on each extract

TABLE VIII

Replicate Analyses and Recovery Experiments on Two Different Extracts of Dried Tomato Leaves

Repli- cate	Extract 1			Extract 2		
	Apparent γ B ₆ per 10 mg. sample			Apparent γ B ₆ per 10 mg. sample		
	No added B ₆	0.1 γ B ₆ per pl.	Recov- ery per cent	No added B ₆	0.1 γ B ₆ per pl.	Recov- ery per cent
1	0.092	0.144	52	0.077	0.107	30
2	0.099	0.140	41	0.062	0.132	70
3	0.097	0.162	65	0.069	0.125	56
4	0.091	0.123	32	0.067	0.124	57
5	0.101	0.142	41	0.063	0.120	57
6	0.082	0.121	39	0.068	0.143	75
7	0.082	0.118	36	0.066	0.120	54
8	0.080	0.123	43	0.065	0.125	60
9	0.075	0.115	40	0.066	0.128	62
10	0.081	0.142	61	0.076	0.148	72
Av.	0.0880 \pm 0.00289	0.1330 \pm 0.00470	45.0 \pm 3.42	0.0679 \pm 0.00158	0.1272 \pm 0.00371	59.3 \pm 4.00

analyzed. Table VIII gives the results of 2 experiments in which 10 replicate recovery determinations were done (with duplicate cultures in each case) on each of two extracts. The recovery values show considerable variation, differing among themselves on the average by 17% of the mean value. The apparent pyridoxin per sample (uncorrected for recovery) shows a smaller variability, varying on the average by 5-9% of the mean. Preliminary assays were therefore carried out in the following manner. Each sample was analyzed in duplicate for apparent pyridoxin content and for each sample a duplicate determination of pyridoxin recovery was made. In each experiment 10 or more similar

samples (as tomato leaves, etc.) were analyzed. Recoveries of related samples were averaged where possible and this average used in computing the pyridoxin content of the individual samples. An additional uniform correction was made for the fact that at most about 85% of the pyridoxin present in the sample is extracted by the method used.

Distribution of Pyridoxin in Tomato Plants. The distribution of pyridoxin in tomato plants was investigated with the aid of the *Neurospora* assay as outlined above. The plants used for the data of Table

TABLE IX

Pyridoxin Concentrations in Various Parts of Tomato Plants, Approximately 40 cm. Tall

Analysis on 5 mg. duplicate samples. Leaves numbered from apex

Plant part	Appar- ent B ₆ per sample	Re- cov- ery	Conc. of B ₆ /g. corr. for av. recov- ery of 66%	Plant part	Appar- ent B ₆ per sample	Re- cov- ery	Conc. of B ₆ /g. corr. for av. recov- ery of 66%
	γ	<i>per cent</i>	$\gamma/g.$		γ	<i>per cent</i>	$\gamma/g.$
Apex (leaves less than 3 cm. long	0.081	69	24.6 ^a	Leaf 6	0.078	68	23.6
Leaf 1	0.143	51	43.4	" 7	0.067	46	20.3
" 2	0.089	50	26.9	" 8	0.066	63	20.0
" 3	0.082	56	24.8	" 9	0.053	69	16.1
" 4	0.075	75	22.7	" 10	0.049	68	14.8
" 5	0.074	87	22.4	Roots (10 mg. sam- ple)	0.092	61	13.9

IX were approximately 40 cm. tall and were greenhouse grown in gravel supplied with nutrient solution. Ten plants were composited and the composite samples dried in a current of air at 60°C., ground in a Wiley mill, and analyzed. Table XI shows that the recovery of added pyridoxin in the presence of the various leaf extracts, while variable, was no more variable than in the experiments of Table VIII, and no trends in recovery are apparent. The mean recovery value was therefore used in computing the pyridoxin contents of the several samples. A general trend of decreasing concentration of pyridoxin from the apex to the base

of the plant is apparent, both in the leaves (Table IX) and in the stem (Table X). In the stem data of Table X, there is a possible trend in the

TABLE X

Pyridoxin in Various Sections of the Stems of Tomato Plants. Approximately 40 cm. Tall

Each experiment based on composite samples from 10 plants

Experiment 56				Experiment 57			
Portion of stem	Appar-ent B ₆ γ/10 mg.	Recov-ery per cent	B ₆ per g. corr. for re-covery	Portion of stem	Appar-ent B ₆ γ/10 mg.	Recov-ery per cent	B ₆ per g. corr. for re-covery
Top	0.106	60	17.7	Top	0.090	52	17.3
2nd	0.065	82	7.9	2nd	0.071	56	12.7
3rd	0.047	95	4.9	3rd	0.060	77	7.8
4th	0.041	102	4.0	4th	0.057	63	9.0

TABLE XI

Accumulation of Pyridoxin at Girdles of the Stems of Petioles of Young Tomato Plants

Samples taken 5 days after girdling in each case

Expt.	Part girdled	% of pls/ treat- ment	Control, not girdled		1.6 cm. sections above girdle		1.6 cm. sec- tions below girdle	
			Re- covery	B ₆ /g. calc.	Re- covery	B ₆ /g. calc.	Re- cov- ery	B ₆ /g. calc.
			per cent	γ	per cent	γ	per cent	γ
61	Stem at 2nd node	96	84	5.8	81	15.1	84	7.5
58	Stem at 2nd node	40	78	6.4	88	15.0	84	6.1
58	Stem at apex be- tween expanding and mature leaves	40	84	9.5	90	10.7	80	15.1
61	Petioles of mature leaves	96	76	9.8	72	25.7	71	10.0
58	Petioles of mature leaves	60	78	9.7	80	21.0	87	5.7

recovery values, and each apparent pyridoxin concentration was there-fore corrected by its own recovery value.

The distribution of pyridoxin in the vegetative tomato plants in so far as it can be judged from these data would appear to conform qualitatively to the distributions previously described for thiamin (Bonner, 1942), riboflavin, and pantothenic acid (Bonner and Dorland, 1943).

Accumulation of Pyridoxin at a Girdle. Young tomato plants (6-12 weeks from planting) similar to those used in earlier work (Bonner, 1942) were girdled variously by application of a jet of steam. Five days after treatment, 1.6 cm. sections of stem or petiole were cut from immediately above or below the girdled region and comparable sections were cut from control, non-girdled, plants. Table XI shows that in 2 experiments pyridoxin appeared to accumulate above a girdle made at the second node, and to be accumulated on the distal side of a girdle made on the petiole of a mature leaf. When stems were girdled near the top of the plant so that only mature leaves were below the girdle and only young rapidly expanding leaves above the girdle, pyridoxin appeared to accumulate below the girdle (Table XI).

The present preliminary data on the distribution of pyridoxin after girdling parallel closely the results obtained in comparable experiments concerning the distribution of thiamin after girdling of tomato plants (Bonner, 1942).

SUMMARY

1. The use of a mutant, obtained by Beadle and Tatum (1941), of *Neurospora sitophila*, for the assay of the pyridoxin content of tomato plants has been described. Extracts of tomato leaves were relatively toxic to the test organism and the assay was unsatisfactory owing to the need for either preliminary purification of the extract or the abundant use of recovery determinations. Despite these difficulties, semi-quantitative results appeared to be obtained. The assay was relatively rapid in that only 48 hours of incubation are needed.

2. The distribution of pyridoxin in the tomato plant resembled the distributions of thiamin, riboflavin, and pantothenic acid which have been previously described. A gradient in pyridoxin concentration from apex to base of the plant was found, with higher concentrations of the substance in the younger leaves and in the top of the stem than in the older leaves and in the base of the stem.

3. Pyridoxin appeared to accumulate above a girdle made by steaming the base of a young tomato plant at the second node. Pyridoxin simi-

larly appeared to accumulate on the distal side of a girdle made by steaming the petiole of a mature leaf of a young tomato plant.

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On the Mechanism of Enzyme Action. Part 21.

Intermediary Phases in the Enzymatic Breakdown of *d,l*-Alanine by *Fusarium lini* Bolley

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INTRODUCTION

No presumptuous dialectics can dispose of the facts, that *e.g.* a) a living cell produces starch from hexoses, while juices obtained from the same cells supply us with glycogen (7b). Or, that b) in the presence of a large excess of added cozymase a normally non-phosphorylating embryo brei (12) breaks down hexosediphosphate. Or, that c) in the course of carbohydrate dissimilation in the central nervous system (10) no phosphorylation of glucose occurs in the white substance on account of lack of hexokinase. In distinction to recent corrections by Van Niel (1), and in accordance with earlier (18) interpretations, we again choose, therefore, the living cell as an impartial agent for the observation of our results.

The study of the mechanism of degradations effected by the enzyme systems present in *Fusaria*, has produced substantial advances within the last few years (7a, 13, 17, 19). However, until now, there still remains an important class of compounds which has not been investigated, namely, the amino acids.

The degradation of amino acids by various microorganisms has been studied (4, 6), but keto acid formation in the course of oxidative deami-

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² Communication No. 28.

nation has been established in only scattered cases, *e.g.*, (2). The finding (19), that nitrates present in the medium (in the form of the potassium or ammonium salt) lend material assistance in the elucidation of the course of the alcoholic fermentation of pentoses and hexoses by *Fusaria*, in distinction to the yeasts (14), was applied to an investigation of this group of compounds, under well characterized experimental conditions.

EXPERIMENTAL

a) Chemical Methods

Keto acids were detected qualitatively by the Lu (11) method for the quantitative determination of pyruvic acid.

Pyruvic acid was determined quantitatively by the method of Fromageot and Desnuelle (5) by oxidation with ceric sulfate. The authors' original procedure was modified by increasing the oxidation period to thirty minutes and substituting ferrous *ortho*-phenanthroline for the ferricyanide indicator recommended. As phosphate interferes in determinations with ceric ions by forming insoluble salts, it was first removed by precipitation as magnesium ammonium phosphate. After its removal by filtration, the solution was strongly acidified with sulfuric acid, and the pyruvic acid content determined. Alanine does not interfere in this procedure.

Hydrogen peroxide was detected by the color reaction of Schales (16).

Alanine was determined by Kjeldahl digestion. The ammonia which was formed by the enzymatic activity of the organism was first removed by boiling the portion of the medium used for this analysis.

Nitrite was detected by the color reaction of Griess, as modified by Blom (3).

Nitrate was determined by precipitation with nitron (15) after first adjusting the pH of the medium to between 1.5 and 2.0.

b) Microbiological Methods

Incubation. The results reported herein were obtained by growing the *Fusaria* in the dark at 28° under aerobic conditions.

Size and type of flasks. All experiments were carried out in 125 ml. Erlenmeyer flasks, 50 ml. of nutrient being pipetted into each one.

Culture employed. The culture used in this investigation was originally obtained from the Biologische Reichsanstalt, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber, and bore the designation No. 5140. Stock cultures of the preceding were maintained on a nutrient medium of the following composition:

20.0 g. glucose
1.00 g. KNO ₃
1.50 g. KH ₂ PO ₄
0.75 g. MgSO ₄
1000 ml. water

and transferred at intervals of two weeks. Microscopic examinations were carried out from time to time to check the purity of the cultures.

Inoculations of flasks were made by adding, with a sterile, hypodermic needle, 1 ml. of a uniform spore-mycelial suspension to each 50 ml. of nutrient medium. This suspension was prepared by growing the fungus on a solid medium contained in a 125 ml. Erlenmeyer flask. The medium for this purpose was the same as above, supplemented by 20.0 g. of agar. After growth had taken place for a period of 8-12 days, 50 ml. of sterile, distilled water were added to each flask containing the solid medium. The flask was then vigorously swirled to remove the spores and some of the mycelium from the plate.

Sterilization was effected by heating the flasks in an autoclave for 20 minutes at fifteen pounds pressure.

c) Method of Calculating and Reporting Results

The contents of four individual flasks were transferred quantitatively to a 250 ml. volumetric flask after filtering off the mycelium. The volume was brought to the mark and an aliquot taken for each of the various analyses which were run in duplicate for the desired constituent. The results were then calculated to signify the amount present in 100 ml. of the original medium. This procedure was adopted to compensate for some evaporation which occurred during the period of incubation and to offset possible inequalities in growth among the several flasks.

d) Results

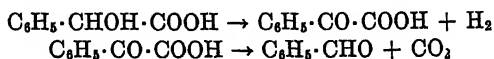
With a nutrient medium of the following composition

20.00 g. *d,l*-alanine
10.00 g. KH_2PO_4
0.75 g. MgSO_4
water to 1000 ml.
pH adjusted to 4.0

it was found possible to identify two of the important intermediates involved in this degradation, namely, pyruvic acid and hydrogen peroxide. The former occurred in isolable amounts (identified by melting point and analysis of its 2,4-dinitrophenylhydrazone) and the latter in traces as would be expected from the findings of Hayasida (9) on the catalase content of *Fusaria*.

In the case of *d,l*-phenylalanine, we have not yet succeeded in definitely characterizing the intermediates involved in its dissimilation but it was observed that a keto acid(s) is present and a non-acidic carbonyl compound(s) accumulates under the experimental conditions chosen. Likewise, in experiments with *d,l*-mandelic acid (which was considered to be of possible help in the solution of this problem) intermediates did not accumulate in quantities sufficient to permit positive identification.

Trapping experiments were likewise without success. For example, one of the possible modes of breakdown of mandelic acid could be the following:



Efforts to trap benzaldehyde with dimedon were unavailing. It appeared obvious that, due to the omnivorous nature of the organism, intermediates on which to base a possible scheme of breakdown, simply were not forthcoming in the respective cultures.

TABLE I
Dissimilation of dl-Alanine

Day	Alanine Present		PA Accumulated		Nitrate Present	
	no KNO ₃	5.00 g. KNO ₃ /l	no KNO ₃	5.00 g. KNO ₃ /l	no KNO ₃	5.00 g. KNO ₃ /l
	g.	g.	mg.	mg.	mg.	mg.
0	2.01	2.01	—	—	—	306
14	0.50	0.62	50	45	—	278
16	0.38	0.55	80	58	—	276
18	0.35	0.36	53	37	—	—

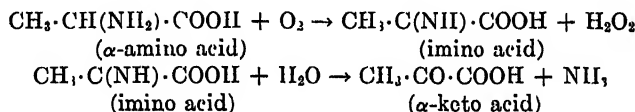
Mycelium Weights—18th day

<i>Without nitrate</i>	<i>With nitrate</i>
176 ± 2 mg.	162 ± 1 mg.

In view of previous results which proved a reduction of nitrate to nitrite, which in turn, caused a partial inhibition of the carboxylase system of *Fusaria*, it was decided to supplement the medium with 5.00 g. of potassium nitrate per liter. From the results recorded in Table I, it can be seen that there is no increased accumulation of pyruvic acid in the presence of nitrate. As a matter of fact, the amount which accumulated was actually smaller. This is very probably due to the slightly diminished rate of dissimilation of the alanine. In the case of the alanine, the lessened accumulation of pyruvic acid in the presence of nitrate is attributable to the *secondary* utilization of the nitrate ion, and, as a matter of fact, the nitrite test was very much weaker (when run on a comparative basis) than in carbohydrate cultures. Moreover, it is obvious that, since far over 50 per cent of the alanine was dissimilated, *Fusaria* are able to attack both optical isomers.

DISCUSSION

On the basis of the results presented, the enzymatic breakdown of *d,l*-alanine by *Fusaria* can be said to follow the course given below,



in accordance with the mechanism usually postulated.

However, it is obvious that another mechanism may be involved, with a hydrolytic deamination resulting in the formation of lactic acid, followed by a dehydrogenation (8) of this compound to yield pyruvic acid. In both cases, the intermediates identified could be the same, and, in fact, hydrogen peroxide was detected, and the presence of a keto acid indicated, when lactic acid served as the sole carbon source.

The findings recorded in this paper lend further experimental confirmation to the previously reported conclusions concerning the mechanism of augmented pyruvic acid accumulation during the fermentation of hexoses and pentoses in the presence of nitrate ions. Furthermore, as presented in our previous scheme (19), they place pyruvic acid in a central position in the entire metabolism of this unique organism: as an intermediate in the degradation of pentoses, hexoses, and alanine, and as a probable intermediate (*via* the reduction of the oxime of the keto acid) in the synthesis of one of the amino acids used in the anabolism of the cell proteins.

SUMMARY

1. Pyruvic acid and hydrogen peroxide were detected as intermediates in the degradation of alanine by living *Fusaria*.

2. Nitrate ions are used only as a *secondary* nitrogen source in the presence of alanine, this fact accounting for the *non*-increased accumulation of pyruvic acid in this degradation.

3. *Fusaria* are able to dissimilate both the *d*- and *l*-forms of this amino acid.

4. The central position of pyruvic acid in the course of the enzymatic degradation of hexoses, pentoses, and amino acids by *Fusaria* is discussed.

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Growth, Ageing, Chronic Diseases, and Life Span in Rats

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INTRODUCTION

The first purpose of the present study was to follow the development of chronic diseases in normal and retarded rats by killing samples of the population at regular intervals. The second purpose was to determine the effect of feeding an adequate basal diet supplemented with four sources of calories commonly eaten by man, namely starch, sugar, milk, and meat.

In the two preceding studies of retarded growth (1) (2) evidence was presented that retarded animals lived for longer periods because they were less subject to such diseases as those of the lungs that attack rats in middle life. Earlier observations had also indicated that retarded rats were usually free from tumors during the period of retardation. In the second of the preceding studies a small number of rats had been retarded for a thousand days without complete loss of the power to grow after realimentation. This observation justified extension to help determine the maximum period of retardation.

EXPERIMENTAL

This experiment started in March 1939 and terminated with the death of the oldest rat in the same month of 1943.

Five hundred rats were started on the experimental diets at the time of weaning. Three hundred of these were retarded in growth. Two hundred others were allowed to grow normally after being divided into four groups.

The same basal diet was fed to all. This consisted of a mixture in grams of cooked starch 20, cellulose 2, cod liver oil 8, lard 7, sucrose 5, alfalfa leaf meal 1, salt mixture 6, dry yeast 14, crude casein 27, and dry liver 10.

The rats retarded in growth were allowed a limited amount of this diet each day. Individuals of the normal groups were given the same daily allowance of the basal diet as the retarded animals plus all they wished to eat of one of the following four supplements depending upon the group.

- | | |
|--|------------------|
| I. Lard 10, Cooked starch 90; | Called "starch." |
| II. Lard 10, Cooked starch 50, Sucrose 40; | Called "sugar." |
| III. Lard 10, Cooked starch 60, Dried whole milk 30; | Called "milk." |
| IV. Lard 10, Cooked starch 60, Dried pork liver 30; | Called "liver." |

In establishing experimental groups, attention was given to both sex and origin of litter.

Most of this experiment was run with the rats housed in an air conditioned room with the temperature at 23°C. Humidity was kept as near 50 per cent as possible. Natural light was excluded from the animal room. Artificial lights were kept on for about an 8 hour working day for the first two years. After this lights were kept on for twelve hours starting at 6 each morning.

Rats were started on the diet at weaning. Retardation was not started until they weighed about 50 grams, each. In general retarded rats were allowed to increase their body weight about 5 grams each 50 days. This increase in weight was made by feeding a supplement of fresh beef liver and fresh lettuce. All control animals were given an equal amount of the same supplement during the same period.

Representative rats from each experimental group were killed at intervals of about 100 days, starting in April, 1939. The bodies of these rats were used for chemical and pathological studies to determine changes in composition and the rate of development of chronic diseases. The results of some of these studies have been published by Lowry and Associates (3). Other studies in this field will appear later by John Saxton, Jr.

In killing animals random selection was used. Five rats of each sex were taken from the retarded animals and one of each sex from each of the four groups that grew normally.

Throughout the study extensive use was made of x-ray photographs for determining the size of the bones at given ages and also for noting the calcification of tissues as the rats grew older.

Starting at 300 days of age representative rats from the retarded group were given all the food they wished in order to allow them to

resume growth. This group, permitted to grow at 300 days, was divided into four sub-groups and fed the diets given the normal rats. Fifty-six rats were used in this group.

After 900 days of retardation another group of twenty rats was allowed to resume growth. At 1100 days, sixteen rats were likewise given an opportunity to resume growth. The poorest rats in the retarded

TABLE I
Life Span of Normal and Retarded Rats

Groups	Mean span <i>in days</i>	Number	Span of three oldest <i>in days</i>
I. Normal "Starch"	Female— 722	19	1065—1103—1117
	Male — 595	19	783— 916— 933
II. Normal "Sugar"	Female— 733	19	932—1147—1154
	Male — 674	19	801— 850— 855
III. Normal "Milk"	Female— 610	19	725— 753— 836
	Male — 688	19	825— 853— 911
IV. Normal "Liver"	Female— 683	19	821— 912— 968
	Male — 531	19	682— 701— 827
Retarded 300 days	Female— 812	28	1147—1167—1196
	Male — 777	24	1023—1048—1054
Retarded 900 days	Female—1124	10	1214—1227—1249
	Male —1086	10	1173—1174—1190
Retarded 1100 days or more	Female—		1320—1368—1456
	Male —		1223—1261—1323
Retarded for entire life	Female —		1311—1320—1456
	Male —		1205—1210—1211

group at this age were selected to determine whether they had lost their power of growth. Many of them died shortly after realimentation.

After 1150 days another small group of ten retarded rats was given adequate food for growth.

Basal metabolism studies were made by Mrs. L. C. Will upon retarded and normal rats in the course of this study. These will be reported separately.

The mean span of life excluding rats sacrificed, from each group is shown in Table I.

The data of Table I indicate clearly the favorable effect of the retardation of growth. Beyond the 900 day period, however, retardation is less favorable. This accords with our earlier experience that 900 days is about the optimum period of retardation for the production of very old rats.

Five per cent of the rats that grew normally exceeded a thousand days in age. These were all females with no male attaining this age. Twenty-

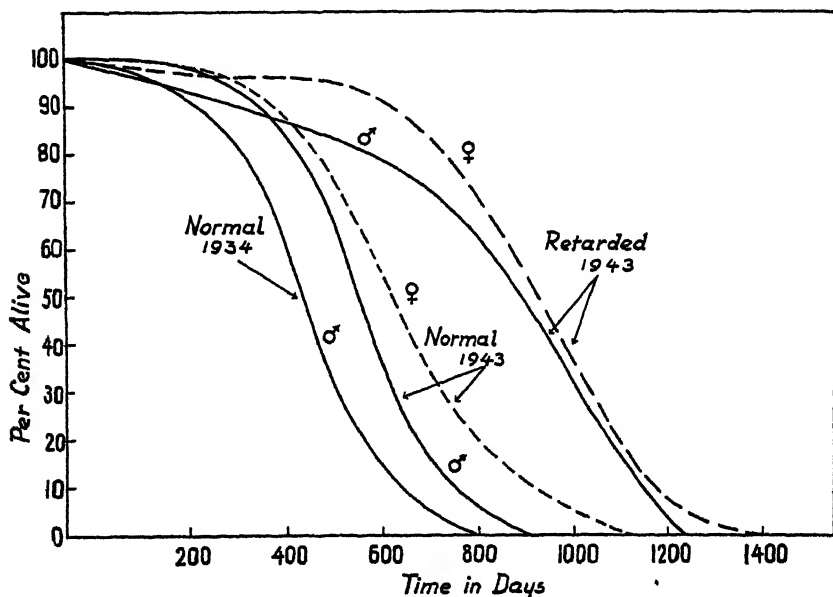


FIG. 1

(Curves Showing the Per Cent of Normal and Retarded Rats Alive at Different Ages

one per cent of the rats that were retarded for 300 days exceeded the age of 1000 days. Four of these were males and seven females. Of the rats retarded in growth more than 300 days, fifty per cent exceeded the age of 1000 days. Forty-seven of these were females and forty were males. The retardation of growth tends to equalize the life span of the opposite sexes. This accords with our earlier experience.

Among the eight groups of normal animals there were only three significant differences that may have been related to the diet. The females fed the "sugar" supplement differed from those given "milk."

The males given the "sugar" and also those given the "milk" supplements lived significantly longer than those fed "liver." In spite of statistical significance we doubt if these differences have real meaning. At least the groups fed the supplements of carbohydrates were not inferior in span of life.

The curves of survivors have been plotted in Fig. 1. The usual difference between the opposite sexes in the case of normal growth is evident. Quite the opposite is true for animals long retarded in growth. Finally the curve for male animals established a decade ago (4) affords comparison and indicates an improvement in survival for normal animals today.

Viability of Spermatozoa

In the course of these studies, when male rats were killed, samples of spermatozoa were examined under the microscope and viability recorded. Among the normal groups motile sperm were found in those individuals fed supplements of "starch" and "sugar," only through the 150 day period. In contrast the rats fed supplements of "milk" and "liver" showed motile sperm when the last group was killed at 620 days of age. Sperm motility persisted among the retarded males through the age of 750 days when this last group was killed.

These results were perplexing inasmuch as all rats received the same daily allowance of the basal diet which must have supplied the vitamin E. Inasmuch as those fed the carbohydrate supplements lost motility of sperm very early while those fed "milk" or "liver" did not, one might conclude that these latter supplements provided an essential supplement of vitamin E. The difficulty in this explanation is that the retarded males retained motility of sperm on the same level of vitamin E that produced sterility in those growing normally with a carbohydrate supplement. Possibly the smaller mass of body tissue in the retarded animals permitted a better economy in the use of vitamin E.

To determine whether the basal diets were deficient in vitamin E, an auxiliary experiment was run. Sixty rats of opposite sexes were fed the basal diet plus the "sugar" supplement. Half of these were given an additional allowance of wheat germ oil. Three hundred and sixty days later, one testis was removed from half of the males with and without wheat germ oil. Those receiving wheat germ oil had motile sperm while the others did not. This seemed to provide evidence that our basal diet was insufficient in vitamin E when the supplement for calories was composed largely of carbohydrates.

When the females from this auxiliary study were about one year old they were bred to normal males. In no case were there litters unless the female had been given the vitamin E supplement.

This experiment with vitamin E is of special interest inasmuch as early sterility in the male as a result of vitamin E deficiency had no effect upon total span of life. The animals from the auxiliary study were retained until the end of life. They also showed no significant differences in life span for the males as indicated in Table II. This is contrary to the common idea that early sterility and premature senility may run parallel, in the male. The females fed wheat germ oil exhibited values for the mean span of life significantly longer than the control group. This was not true in the data of Table I, however. The data are not strictly comparable since several other factors than vitamin E were involved.

TABLE II

Life Span of Rats With and Without Wheat Germ Oil as a Supplement

Variable	Rats No.	Mean life span in days	Mean P.E.
Males with wheat germ oil supplement.....	15	741	39
Males with no supplement.....	15	703	24
Females with wheat germ oil supplement.....	14	840	33
Females with no supplement.....	15	694	26

Oestrous Cycle and Retarded Growth

Vaginal smears were made at intervals on all animals. These were usually made for a period of 21 days and those rats showing no cornified smears were considered anoestrous. As previously found (5) some of the controls showed continuously cornified smears at some time. Also the percentage of anoestrous smears increased gradually with age (Table III).

In the case of retarded rats the results also agree with the earlier ones. In some of the retarded rats (16 out of 48) the vagina did not open until between the 370-375th day.

Normal cycles were reestablished in rats realimented early in life (300 days) 100 per cent. After this it did not have so great an effect but as shown previously it did affect part of the animals.

Pathology Study

Since all pathological findings from this study will be published elsewhere by Dr. John Saxton, Jr., who was responsible for this part of the work, only brief summaries will be included here.

Inasmuch as typical animals were killed starting when relatively young, a picture of the development of the chronic diseases of rats at different ages was completed. Rats retarded in growth exhibited a much greater resistance to these diseases than those that grew normally. The diseases appeared ultimately, especially in those groups allowed to complete their growth. However, at a given age the incidence of chronic disease was much lower in retarded animals than in the controls that grew normally. This accords with our earlier experience. Furthermore, the development of tumors was negligible in rats that were retarded in growth until after they had been allowed to attain maturity.

TABLE III
Changes in the Oestrous Cycle

Age	Controls			
	Alive No.	Anoestrous per cent	Normal per cent	Partly corn per cent
1 year-1 year, 2 months.....	75	5	77	10
1 year, 7 months.....	54	33	46	15
2 years, 7 months.....	6	50	50	—
Raised at 300 days				
1 year-1 year, 2 months.....	28	0	85	—
1 year, 7 months.....	24	12.5	70	16
2 years, 7 months.....	7	14	28	—
Retarded 900 days or more				
1 year-1 year, 2 months.....	100	93	3	—
1 year, 7 months.....	94	100	—	—
2 years, 7 months.....	51	17.6	41	—

(Ones not accounted for in the above were doubtful.)

The absence of early signs of the common chronic diseases that attack rats indicates the major reason for the extension of the mean span of life.

Two reviews have summarized recent evidence indicating that excess ingestion of nutrients may have an unfavorable effect in relation to disease resistance in animals. The first of these concerns resistance to inoculated tumors in studies such as those of Tannenbaum using low calory diets (6). The second deals with the studies of Claire Foster and others in showing decreased resistance of mice to poliomyelitis when fed a slight excess of vitamin B₁ (7) (8). Likewise these mice had greater resistance when fed low calory diets. These findings accord with our observations in all studies of retarded growth.

Several attempts have been made by us to learn more about the cause

of the chronic lung disease that afflicts rats during the latter half of life. Some years ago we attempted to isolate specific organisms but failed. In the course of the present study an auxiliary experiment was run to determine whether either air conditioning of the rat quarters or the relative dustiness of the feed might affect the resistance of the lungs. Since this study will be described later by Dr. John Saxton, Jr., only a brief summary is included here.

Eighty rats were divided into four groups. Half of these rats were kept in air conditioned quarters and half in a room subject to the usual variable conditions in a laboratory. These rats were further subdivided so that half received a dry, rather dusty, mixture of stock diet while the other half had this same diet well moistened. These rats were autopsied after the experiment had been in progress for 500 to 600 days.

The males showed less resistance to the common lung disease than the females. This accords with the common experience that the females live longer. The difference in relation to the variables studied were not great enough to be significant. Therefore, it is unlikely that we have discovered factors responsible for the lung disease although animals retarded in growth are much more resistant than normal ones. Neither air conditioning nor the relative dustiness of the diet seems to be an important factor in producing diseased lungs.

Growth

In our earlier studies the resumption of growth after 900 days of retardation was achieved easily but after 1000 days some rats succeeded and part failed. Under the conditions of these experiments the thousand day period seems to be a crucial dividing line. In the present trials the ability to resume growth has been judged by changes in body weight and by measurements of bone length. These latter are based upon x-ray photographs taken at regular intervals throughout the experiment. Details will be presented in a later report.

In this study there was no question that growth was resumed at 900 days of age. The group retarded for 1100 days and then allowed to resume growth was selected from the rats in the poorest health since it was assumed if these could resume growth that the remainder of the group could do so. Since this poor group failed for the most part, a healthier fraction was chosen for realimentation at 1150 days of age. The response of this group in terms of weight increase is given in Table IV. This indicates clearly that some individuals but not all can resume growth after 1150 days of retardation.

TABLE IV

Changes in Retarded Rats Allowed to Grow after 1150 Days of Age

Rat No.	Sex	Wt. at 1150 days g.	Max. wt. attained g.	Tibia length at 1150 days cm.	Max. tibia length cm.
3	Fem.	162	176	3.27	3.29
96	Fem.	147	156	3.19	3.19
110	Fem.	160	165	3.19	3.20
120	Fem.	164	180	3.17	3.20
191	Fem.	161	197	3.30	3.32
12	Male	155	Died	3.43	—
128	Male	165	229	3.33	3.43
150	Male	161	166	3.29	3.30
154	Male	162	196	3.31	3.35
250	Male	160	198	3.30	3.34

TABLE V

Ultimate Body Size After Retarded Growth

Normal Growth		No. of An.	Mean Max. Wt. Attained g.	Extremes of Max. Wt. Attained g.	Age of Max. Wt. days	Tibias No.	Mean tibia length at 348 days cm.
I. F.	Starch	19	277	192-359	473	6	3.55
M.		19	381	284-471	430	6	3.78
II. F.	Sugar	19	303	229-417	468	7	3.59
M.		19	445	305-543	458	6	3.89
III. F.	Milk	19	330	229-458	422	6	3.60
M.		19	515	399-677	454	—	4.06
IV. F.	Meat	19	324	217-521	472	6	3.63
M.		19	460	281-600	391	6	4.01
Retarded 300 days							At age 530 days
F.		28	270	168-373	594	10	3.44
M.		24	351	272-475	609	10	3.73
Retarded 900 days							At age 1050 days
F.		10	193	158-223	982	8	3.39
M.		8	248	219-283	1007	8	3.53

Ultimate Body Size

The rat seems to be the species least stunted permanently by long retardation of growth. In the past, however, evidence has accumulated

that even the rat is somewhat dwarfed by a relatively short period of retardation. Some pertinent data from these trials have been assembled in Table V.

Definite conclusions can be drawn in regard to ultimate body size. The male exceeds the female even after 900 days of retarded growth with maintenance of the opposite sexes at about the same weight during retardation. The close correlation between the length of the tibia and the gross body weight indicates that the weight of the body is a better criterion of true size than is usually supposed. In other words the degree of fatness is less misleading or perhaps more constant than one imagines, when groups of rats are considered.

Response to a Dietary Regime After the Retardation of Growth

In the present study the group of rats allowed to resume growth after 300 days of retardation was divided into four sub-groups. These were placed upon the same major calory supplements allowed the four normal groups, namely "starch," "sugar," "milk," and "liver" as indicated at the beginning. The response as indicated in the growth curves which are not reproduced here was in the same order as that of the original groups of rats that had not been retarded in growth.

SUMMARY

In a third study of retarded growth in relation to ageing, rats were killed at regular intervals to determine the incidence of the common chronic diseases that usually terminate life prematurely. Retarded rats are much less subject to these diseases than those that grow normally when groups equal in age are considered.

Rats can be retarded as much as 1150 days and still resume growth when provided with adequate calories. This resumption of growth was followed by weight increases and x-ray photographs of bones.

Rats were allowed to grow normally with an adequate allowance of a good basal diet plus supplements of interest in human nutrition, namely milk, meat, starch, and sugar as additional sources of calories. It is doubtful if these four supplements modified the total span of life significantly. With equal amounts of vitamin E rats on the richer carbohydrate diets tended to become sterile early in life in contrast to retarded animals upon the same allowance. This premature sterility in the males had no effect upon the total span of life.

Rats retarded for 300 days responded to the four additional sources

of calories in the same order as those allowed to grow normally from the beginning. Such rats were unable to attain the same body size as controls not subjected to retardation.

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Ageing, Basal Metabolism, and Retarded Growth

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INTRODUCTION

This study of the basal metabolism of the rats of the retarded growth experiment conducted by McCay and co-workers was undertaken to compare the heat production of rats retarded in growth with that of rats of the same age which had been allowed to grow normally. Since the rats studied were over 800 days of age, the data may also be useful as a supplement to the knowledge of the basal metabolism of old rats.

Ashworth and associates (1) in studies with retarded rats found that body weight was the most influential factor in limiting the metabolic rate and that age was relatively negligible. They suggested that the higher basal metabolism (per unit of weight) of underfed animals might be attributed to the fact that in such animals the viscera constituted a greater proportion of the body weight.

Horst and others (2) found that on the basis of surface area, retarded rats had a lower basal metabolism than either weight or age controls. These workers also observed that the basal metabolism of rats remained quite constant during the second and third years of life.

In a study with rats up to about 1100 days of age, Benedict and Sherman (3) noted a slight decline in total heat production during later life. Since body weight declined somewhat more than heat production there was an apparent increase in metabolism per unit of body weight.

Davis (4) found that the oxygen consumption of rats decreased throughout life, rapidly during the first four months and more gradually thereafter.

In 1939, Alex Black (5) observed a wide variation in the basal metabolism of a group of old male rats whose average age equalled 711 days. In comparisons between animals of equal weights, a higher basal metabolism was observed in the older animals.

EXPERIMENTAL

The plan of the retarded growth experiment and details have already been described in the preceding paper by McCay, Sperling, and Barnes. Basal metabolism determinations were made on a number of the experimental animals. Twenty-seven control rats were studied, 9 males and 18 females, between 800 and 900 days of age. Seven rats (4 males, 3 females) were observed which had been realimented after a retardation period of 900 days. Five animals (3 males, 2 females) were tested which had been realimented at 1150 days of age. The two last named groups were about 1200 days old at the time the metabolism measurements were made. Twenty-two retarded rats (11 males, 11 females) were studied between the ages of 800 and 900 days. Eleven retarded rats (5 males, 6 females) were observed after they had reached 1200 days of age. All animals studied appeared to be in good health at the time the tests were made.

It was planned to repeat the basal metabolism determinations on each individual until results were obtained which agreed within one calorie per square meter per hour. This checking was carried out for most of the animals, but some individuals became sick or died before the check determinations were made.

The metabolism apparatus employed was that described by Forbes, Kriss, and Miller (6). Temperature, light, and ventilation rate were maintained as uniformly as possible throughout the experimental period. By means of an automatic thermo-regulator, the air bath was kept at 30°C. which, according to the work of Swift and Forbes (7) is the critical temperature for the albino rat. The ventilation rate was maintained at 1 to 2 liters per minute. Bright illumination was used to discourage activity during tests.

The animals were brought to a post-absorptive state by a fast of 15-17 hours. The carbon dioxide and moisture production were measured over a six-hour period. The amount of activity was recorded hourly by means of a work adder attached to the respiration chamber. In calculating the average hourly carbon dioxide production, the carbon dioxide eliminated during the first hour was omitted. This procedure allowed the animals to become accustomed to the chamber. Thus, temperature equilibrium was established. This also allowed for accumulation of carbon dioxide in the chamber during the weighing period. If the work adder recorded unusual activity during some part of the test, the value for that hour was omitted.

Diack's formula (8) for the surface area of fasting albino rats was used for the retarded animals:

$$S = 7.64 \times wt.^{2/3}$$

For the surface area of the control rats and for the surface area of those rats which were allowed to grow normally after a certain period of retardation, Diack's formula for normal rats was used:

$$S = 7.47 \times wt.^{2/3}$$

In both normal and retarded animals no differences in basal metabolism related to sex were found.

TABLE I

Basal Metabolism of Normal and Retarded Rats at an Age of 800 to 900 Days

<i>Group</i>	<i>Sex</i>	<i>No.</i>	Mean Basal Metabolic Rate <i>cal./m.²/hr.</i>	<i>cal./kg/hr.</i>
Retarded	Male	11	29.6 ± 0.67	4.50 ± 0.10
Control	Male	9	32.4 ± 0.69	3.41 ± 0.09
Retarded	Female	11	30.5 ± 0.69	4.63 ± 0.09
Control	Female	18	33.0 ± 0.35	3.83 ± 0.07
Retarded	Male and female	22	30.1 ± 0.47	4.57 ± 0.07
Control	Male and female	27	32.8 ± 0.32	3.69 ± 0.06

At about 850 days of age retarded rats had a higher basal metabolism than controls (Table I) in terms of calories per kilogram of body weight. The basal metabolism of retarded rats per unit of weight was significantly higher than that of animals retarded for 900 days and then realimented for a period of 300 days. The comparison was made when both were about 1200 days of age (Table II). Thus the same relation seems to exist that has been found when retarded rats are compared with normal ones much earlier in life. In contrast, those realimented at 1150 days, showed no differences from retarded animals. This was probably a reflection of the failure to achieve normal basal metabolism after this extreme period of retardation.

Retarded rats whose basal metabolism was measured at 850 and 1150 days of age gave the same values. In this case, therefore, there was no evidence for a change of basal metabolism related to age.

When calculations were based on surface area, the values for retarded

rats at 850 days of age were significantly lower than those for controls. Also on this basis there was no difference between rats realimented at 900 or 1150 days of age and retarded animals of the same age. In contrast to data based upon body weight, those calculated to surface

TABLE II

Basal Metabolism of Retarded and Realimented Rats at 1200 Days of Age

Group (Males and Females)	No.	Mean Basal Metabolic Rate cal./m ² /hr.	cal./kg./hr.
Retarded	11	33.1 ± 0.56	4.68 ± 0.08
Realimented at 900 days	7	32.5 ± 0.47	4.23 ± 0.08
Retarded	11	33.1 ± 0.56	4.68 ± 0.08
Realimented at 1150 days	5	35.8 ± 1.84	4.77 ± 0.26
Realimented at 900 days	7	32.5 ± 0.47	4.23 ± 0.08
Realimented at 1150 days	5	35.8 ± 1.84	4.77 ± 0.26

TABLE III

Basal Metabolism of Retarded Rats at Two Different Ages

Age Days	Sex	No.	Mean Basal Metabolic Rate cal./m ² /hr.	cal./kg./hr.
850	Male	11	29.6 ± 0.67	4.50 ± 0.10
850	Female	11	30.5 ± 0.69	4.63 ± 0.09
1200	Male	5	32.6 ± 0.86	4.61 ± 0.12
1200	Female	6	33.6 ± 0.78	4.74 ± 0.11
850	Male	11	29.6 ± 0.67	4.50 ± 0.10
1200	Male	5	32.6 ± 0.86	4.61 ± 0.12
850	Female	11	30.5 ± 0.69	4.63 ± 0.09
1200	Female	6	33.6 ± 0.78	4.74 ± 0.11
850	Male and female	22	30.1 ± 0.47	4.57 ± 0.07
1200	Male and female	11	33.1 ± 0.56	4.68 ± 0.08

area show a lower basal metabolism for retarded rats at 850 than at 1200 days of age.

In general our values accord with those of others for rats of similar age. As far as we are aware these are the first measurements upon rats that have attained the age of 1200 days after long periods of retardation.

SUMMARY

1. Retarded rats at 850 days of age had a significantly higher heat production per unit of weight than normal rats; and a significantly lower heat production per unit of surface area than these controls.

2. Retarded rats at 1200 days of age had the same basal metabolism per unit of surface area as rats realimented at either 900 or 1150 days. Per unit of weight, retarded rats did not differ from rats realimented at 1150 days, but had a higher heat production than rats realimented at 900 days.

3. Retarded rats at 1200 days of age had a higher heat production per unit of surface area than retarded rats at 850 days. Heat production per unit of weight was not significantly different.

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Mode of Action of Some Antibacterial Mould Products

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INTRODUCTION

In addition to penicillin, a variety of antibacterial agents has been obtained from cultures of *Penicillium notatum*. Penatin was described by Kocholaty (1, 2) as a protein with a far wider range of bacteriostatic and bactericidal activity than penicillin. The purest preparations are light yellow powders, readily adsorbed by Kaolin, quite resistant to acids, but inactivated in alkaline solution. The mode of action does not seem to be lytic. Penatin requires glucose in order to be effective, and it will decompose this carbohydrate with the formation of an unidentified acid and hydrogen peroxide.

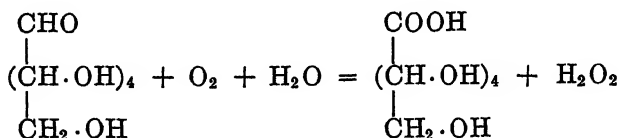
Penicillin B has been isolated by Doisy and collaborators (3) as a protein which is highly effective against both Gram-negative and Gram-positive organisms. The purest preparations are again light yellow powders, soluble in water, insoluble in organic solvents and precipitated from aqueous solution by two-thirds saturation with ammonium sulfate. Penicillin B also requires glucose in order to exert its antimicrobial action. If galactose is used instead, complete inhibition of the test organisms is obtained only if eight times as high a concentration of Penicillin B is present.

Notatin, isolated by Raistrick, *et al.* (4) is a very powerful bactericide in the presence of oxygen and glucose and in the absence of appreciable amounts of catalase. The British workers identified notatin as an antibacterial glucose-aerodehydrogenase, *i.e.*, a yellow enzyme.

According to Kocholaty (2) the mode of action of these three mould products is not understood. It is the purpose of this paper to offer a simple explanation for their antibacterial activity.¹

¹ After this paper had been written, Doisy, *et al.* (9) and Birkinshaw and Raistrick (10) published findings which lead to conclusions identical with the ones arrived at here.

In connection with earlier work on sensitive methods for the detection of hydrogen peroxide and its formation in certain dehydrogenation processes (5), we became interested in glucose oxidase. This enzyme, which has been the subject of extensive studies by Franke and collaborators (6, 7), is present only in moulds. It oxidizes glucose to gluconic acid:



Glucose oxidase is a yellow (flavin) enzyme (7), soluble in water, insoluble in organic solvents, precipitated by 0.7 saturation with ammonium sulfate and adsorbed on kaolin. Properties and isolation methods described for glucose oxidase are very similar to those of the three antibacterial mould products. While notatin has already been identified as a powerful glucose-aerodehydrogenase producing gluconic acid and hydrogen peroxide (4), it seems very probable in view of the known facts that penatin and penicillin B are also glucose oxidases.

The action of glucose oxidase on glucose produces hydrogen peroxide which can be demonstrated easily with the luminol- and the phenolphthalin tests (5). Quantitative determinations with Franke's enzyme showed hydrogen peroxide concentrations between 0.005 and 0.01%, when the enzyme in a dilution of 1:10 000 had acted for one hour at 30°C. on a 12 per cent, glucose solution.

The question then arose, whether perhaps the amount of hydrogen peroxide produced by glucose oxidase is sufficient to exert an antimicrobial effect. This seemed unlikely, as H_2O_2 is not considered to be a powerful antiseptic. Dittmar, *et al.* (8) found that a hydrogen peroxide concentration of 0.125% is necessary in order to kill *Staphylococcus aureus* after an exposure of 25 minutes. However, the same authors found also that the H_2O_2 concentration can be decreased to 0.007% under otherwise identical conditions, if optimal amounts of catalysts such as ferric and cupric ions are present.

EXPERIMENTAL

No data could be found on the bacteriostatic effect of H_2O_2 . We did therefore the following experiments in order to determine the minimal concentration of hydrogen peroxide capable of delaying the growth

of *Staphylococcus aureus*. A fresh culture of *Staph. aureus* was diluted with plain broth to about 5000 organisms per ml. Ten ml. of this broth were transferred to each of a number of tubes containing hydrogen peroxide (1 ml.) in various concentrations. The mixtures were incubated at 37°C. Results of a typical experiment are given in Table I.

The antibacterial effective hydrogen peroxide concentrations are well below those easily obtained with glucose oxidase. It should be pointed out also that the enzyme produces hydrogen peroxide continuously and at a constant rate, as long as glucose is available in excess. In our experiments the conditions are different and less favorable for the suppression of bacterial growth. Here, the H_2O_2 concentration is highest

TABLE I

Effect of hydrogen peroxide on growth of Staph. aureus

(No visible growth: —; growth indicated by + to +++)

% H_2O_2 * in mixture	Incubation time in hours						
	4	5	10	24	28	48	72
0.107	—	—	—	—	—	—	—
0.011	—	—	—	—	—	—	—
0.0054	—	—	—	—	—	—	—
0.0027	—	—	—	—	—	—	—
0.0021	—	—	—	—	—	—	++++
0.0016	—	—	—	+++	++++	++++	++++
0.0011	—	—	+	++++	++++	++++	++++
0.0005	—	—	++	++++	++++	++++	++++
0.0003	—	—	++	++++	++++	++++	++++
0.0000	—	+	++	++++	++++	++++	++++

* C P. Baker's analyzed, special.

at the beginning of the test and decreases gradually while the oxidizing agent is in contact with organic material. The sensitive luminol test for hydrogen peroxide (5) for example was negative, after broth containing 0.0027% H_2O_2 had been incubated for 8 hours.

As can be seen from Table I as little as 0.0003% hydrogen peroxide delays bacterial growth. From 0.0027% H_2O_2 on and higher, the action is bactericidal, as revealed by subcultures done 24 hours after the start of incubation with peroxide.

From these results it seems very probable that the antibacterial effect of the three mould products under discussion is due to the hydrogen peroxide produced by them. One is perhaps even justified in postulating the hypothesis that under proper conditions any enzyme which will

LETTER TO THE EDITORS

Rôle of Phosphate in the Anaerobic Dissimilation of Pyruvic Acid

Inorganic phosphate has been observed to participate in several reactions involving pyruvic acid. Lipmann (1940) found that a preparation of *Lactobacillus delbrueckii* which oxidized pyruvic acid to acetic acid and CO_2 , formed adenosine triphosphate from adenylic acid and inorganic phosphate as a part of the reaction. Lipmann also described a labile phosphate compound, acetyl phosphate, as an intermediate in the reaction. Banga, *et al.* (1939) working with brain extracts found that adenosine triphosphate was synthesized during the oxidation of pyruvic acid. However, Ochoa, *et al.* (1939) found brain dispersions unable to oxidize acetyl phosphate and concluded this compound was not an intermediate. Still (1941) obtained a cell-free preparation of *Escherichia coli* which needed phosphate for the oxidation of pyruvate. Koepsell and Johnson (1942) obtained a cell-free preparation from *Clostridium butylicum* which converted pyruvic acid to acetic acid, CO_2 , and H_2 . This preparation needed phosphate for its action but acetyl phosphate was not detected as an intermediate. Kalnitsky and Werkman (1943) found that phosphate was necessary for the so-called "hydroclastic" reaction as performed by a cell-free preparation of *E. coli*. In this reaction pyruvic acid is converted anaerobically into acetic acid and formic acid.

Further investigation of the "hydroclastic" reaction has disclosed that adenosine triphosphate is synthesized from adenylic acid and inorganic phosphate as a result of the process. A compound resembling acetyl phosphate has been detected as an intermediate in the reaction.

An enzyme preparation was obtained by grinding *E. coli* with powdered glass as described previously (Utter and Werkman, 1942) which gave the following results in the conversion of pyruvate to formate and acetate. The column labelled "acetyl P" was determined according to the method of Lipmann (private communication) in which the acid labile P is determined as the difference between P as precipitated by a CaCl_2 -ethanol mixture at a neutral pH (true inorganic P) and the inorganic P in a similar aliquot as determined by the method of Fiske and Subbarow (1925) in

Cup No.	Contents	Time, Min.	Inorg. P	Acetyl P	Adenosine Triphosphate	Pyruvate Utilized
1	Pyruvate	0	4.94	—	—	—
2	Pyruvate	60	4.08	0.59	0.26	2.29
3	Pyruvate and adenylic acid	0	4.94	—	—	—
4	Pyruvate and adenylic acid	75	3.80	—	1.29	2.00

Values in M moles $\times 10^{-2}$ per cup. Each cup contained 0.8 ml. of enzyme preparation, 0.045 M NaHCO_3 , 0.03 M pyruvate, and additions to a total volume of 2.0 ml. Cups 3 and 4 contained 0.02 M adenylic acid. The experiment was carried out in Warburg manometers at 30.4°C. under an atmosphere of 90% N_2 and 10% CO_2 . The reaction was stopped at the indicated time by ice-cold CCl_3COOH .

Considerable acid-labile P, presumably acetyl P accumulated in the absence of adenylic acid and when the latter was added as a P-acceptor, adenosine triphosphate accumulated. The ratio of P fixed to pyruvate utilized was not unity but experiments are now in progress to determine whether this is the case under proper conditions. Lipmann (1940) found that approximately one mole of acetyl P was formed for each one-half mole of O_2 utilized which would give a ratio of approximately one mole of P per mole of pyruvate oxidized.

The synthesis of adenosine triphosphate during the "hydroclastic" reaction is of interest in that the process is anaerobic in contrast to most of the other reactions involving such synthesis.

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